

AE/1648
Jfw

PATENT

Applicant: Adriano Aguzzi *et al.*

Group Art Unit: 1648

Serial No.: 09/554,567

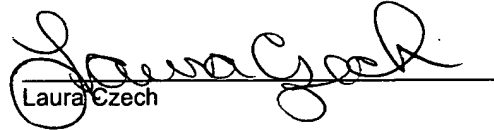
Certificate of Mailing:

Filed: September 1, 2000

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being sent by first class mail to the Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450 on October 4, 2006.

For: Diagnostics and Therapeutics for Transmissible Spongiform Encephalopathy and Methods for the Manufacture of Non-Infective Blood Products and Tissue Derived Products

Attorney Docket No.: ABB01207P00380US;
6458.US.01


Laura Czech

Examiner: Ulrike Winkler, Ph.D.

RE-SUBMITTED APPEAL BRIEF

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

This Appeal Brief is being re-submitted pursuant to the Notification of Non-Compliant Appeal Brief mailed on September 6, 2006. If any additional fees are required as a result of the filing of this paper, the Commissioner is hereby authorized to charge Deposit Account No. 23-0785 for any such fees.

Real Party in Interest

The University of Zurich is the real party in interest in this appeal.¹

Related Appeals and Interferences

There are no related appeals, interferences or judicial proceedings known to Appellant, Appellant's legal representative, or assignee which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in this pending appeal.

Status of Claims

Claims 1-34 and 38-40 have been canceled. Claims 35-37 are rejected and are the subject of this appeal.

Status of Amendments

There have been no amendments made to the claims or specification subsequent to the mailing of the final Office Action on September 21, 2005.

Summary of Claimed Subject Matter

The present invention relates to Transmissible Spongiform Encephalopathy (hereinafter "TSE"). TSE comprises a group of slow degenerative diseases of the

¹ The assignment recorded on September 11, 2000 at reel/frame 011156/0452 contains an error. The recordation sheet says that the assignment was made from the inventors to Abbott Laboratories, Inc. However, the actual assignment is to the University of Zurich.

central nervous system, such as Creutzfeld-Jakob disease (hereinafter "CJD") in man or Bovine Spongiform Encephalopathy (hereinafter "BSE") in cattle, which is also known as "mad cow disease". Specifically, the present invention relates to methods of identifying TSE-infected B-cells and T-cells in a test sample.

It is believed that TSE is caused by the pathogenic agent known as a "prion." It is known in the art that prions are devoid of nucleic acid and are identical with PrP^{Sc} , which are the disease or modified forms of the normal host protein, PrP^{C} (See, the specification, page 2, lines 23-27). PrP^{Sc} is a protease-resistant form of PrP^{C} . So far, no chemical differences have been detected between PrP^{Sc} and PrP^{C} .

As mentioned briefly above, the present invention is directed to methods of identifying TSE-infected B-cells and T-cells. Applicants were the first to determine the roles of different components of the immune system by using a panel of immune-deficient mice inoculated with prions. Specifically, Applicants have discovered that differentiated B-cells are crucial for neuroinvasion by TSE while T-cells play a secondary role in TSE infectivity. Based on these findings, assays according to the present invention are provided which contemplate the monitoring of biological or biochemical parameters of B-cells and/or T-cells to determine the occurrence of TSE infection. The invention takes advantage of the fact that, as opposed to PrP^{C} , PrP^{Sc} is resistant to proteinase K digestion. Because of this fact, any form of PrP^{C} remaining in the B-cells and/or T-cells after digestion with proteinase K will necessarily be PrP^{Sc} .

Therefore, if the cells are exposed to anti-PrP^C antibodies, the antibodies will recognize only abnormal PrP^{Sc} proteins, thus indicating TSE.

Three independent claims are involved in this Appeal, namely, claims 35, 36, and 37. Each of these claims recites virtually identical steps of identifying TSE-infected cells. The difference between the claims is that claim 35 is directed to the identification of TSE-infected B-cells, claim 36 is directed to the identification of TSE-infected T-cells, and claim 37 is directed to the identification of TSE-infected B-cells and T-cells.

More specifically, the claimed methods comprise the steps of:

- obtaining a test sample suspected of TSE infection (See, page 46, lines 6-8, page 52, lines 25-27, page 86, lines 1-2 and original claim 23);

- collecting B-cells and/or T-cells from the test sample (See, page 46, lines 6-8, page 52, lines 25-27 and original claim 23);

- subjecting said B-cells and/or T-cells to homogenization (See. page 82, lines 6-12);

- subjecting said homogenized B-cells and/or T-cells to proteinase K digestion (See, page 46, line 10, page 52, line 30, page 112, lines 27-28 and original claim 23);

- subjecting said digested B-cells and/or T-cells to SDS Page immunoaffinity chromatography blots (See, page 52, line 29, page 112, line 26 and original claim 23);

-contacting said blots with an anti-PrP antibody (See, page 46, line 9, page 52, line 29, page 112, line 30, see original claim 23),

wherein in the presence of a signal from the anti-PrP antibody-PrP complex in the sample is indicative of TSE-infected B-cells and/or T-cells (See, page 46, lines 4-10, page 52, lines 24-30, Figure 10 and page 112, lines 30-34 through page 113, line 1);

-identifying TSE-infected B-cells and/or T-cells based on the presence of said signal (Figure 10 and page 112, lines 30-34 through page 113, line 1); and

-wherein the identification of TSE-infected B-cells is associated with TSE promulgation and primary infection (claim 35), the identification of TSE-infected T-cells is associated with TSE promulgation and secondary infection (claim 36), or the identification of TSE-infected B-cells is associated with TSE promulgation and primary infection and the identification of TSE-infected T-cells is associated with TSE promulgation and secondary infection (claim 37) (See, page 30, lines 3-24, page 35, lines 15-21, page 56, lines 5-10 and page 66, lines 5-9).

Grounds of Rejection to be Reviewed on Appeal

There is only one outstanding ground of rejection that is the subject of this Appeal. Specifically, claims 35-37 are unpatentable under 35 U.S.C. §103(a) as being unpatentable over O'Rourke et al., (U.S. Patent No. 6,165,784) (hereinafter "O'Rourke") and/or Korth et al., (*Nature*, 390: 74-77 (November 6, 1997)) (hereinafter "Korth"), in view of Kuroda et al., (*Infection and Immunity*, 41:154-61 (1983)) (hereinafter "Kuroda") and/or Manuelidis et al., (*Science*, 200: 1069-1071 (1978)) (hereinafter "Manuelidis").

Argument

Rejection under 35 U.S.C. Section 103(a) in view of O'Rourke and/or Korth in view of Kuroda and/or Manuelidis

The Examiner rejected claims 35-37 as being unpatentable over the combination of the teachings of O'Rourke and/or Korth in view of Kuroda and/or Manuelidis.

As discussed above, each of the three independent claims in this Appeal recite virtually identical steps of identifying TSE-infected cells. The difference between the claims is that claim 35 is directed to the identification of TSE-infected B-cells, claim 36 is directed to the identification of TSE-infected T-cells, and claim 37 is directed to the identification of TSE-infected B-cells and T-cells. Therefore, given the similarity between the claims, the patentability of these claims will be argued together as a group.

According to the Examiner, Kuroda teaches that both B-cells and T-cells can transmit TSE, and Manuelidis teaches the importance of focusing on these cellular populations to increase the sensitivity of assays for TSE infectivity. The Examiner further maintains that both O'Rourke and Korth disclose using antibodies as a method of detecting the disease-causing agent. The Examiner alleges that it would have been obvious to apply the techniques taught by O'Rourke and/or Korth to the infected tissue disclosed by Kuroda and/or Manuelidis in order to improve the sensitivity of the TSE tests by collecting samples containing B-cells and/or T-cells. The Examiner finds the

motivation to combine these references in avoiding having to utilize live animals to test for infectivity in the B-cells and/or T-cells.

The Examiner's rejection is erroneous for the following reasons: 1) neither Kuroda nor Manuelidis explicitly teach that B-cells and/or T-cells can transmit TSE; 2) there is no motivation to combine these references; and 3) the Korth reference teaches away from the Applicants' invention.

A. Neither Kuroda nor Manuelidis teach that B-cells and/or T-cells can transmit TSE

Throughout the prosecution of this application, the Examiner has maintained that Kuroda teaches that both B-cells and T-cells can transmit TSE and that Manuelidis teaches that it is important to focus on these cellular populations to increase the sensitivity of assays for TSE infectivity. These statements are overly broad and not entirely correct.

Kuroda reported the results of a study in BALB/c mice that he thought were infected with a Japanese strain of a CJD (a variant of TSE) **virus**. Kuroda detected the "virus" in the brain, spleen, lung, thymus, liver, kidney and blood (but not the urine) of the infected mice at various periods in time after inoculation. Of the examined tissues, the highest infectivity was found in the brain and spleen. Kuroda examined the spleen cells to determine what types of cells were actually infected with the virus. Initially, he found that spleen macrophages, T-cells, and B-cells were infected. Of these cells, the

highest concentration of the virus was found in the lymphocyte fraction. When Kuroda examined the infectivity of various subpopulations of lymphocytes from these “virus”-infected mice, he found that large lymphocytes or blastoid cells in the lower-density fractions from the spleen exhibited the most infectivity.

Accordingly, Kuroda mistakenly believed that CJD was caused by a virus. The very point of the study was to “obtain data on the mode of replication and temporal distribution of CJD virus in relation to onset of the disease and to determine the important implication of the hematogeneous route of virus dissemination” (See, Kuroda, page 154, 1st and 2nd paragraphs). It was later determined that TSE is the result of an abnormal prion, not a virus. Nowhere does Kuroda disclose or suggest that B-cells and/or T-cells transmit TSE.

Second, like Kuroda, Manuelidis also mistakenly believed that CJD was caused by a virus (See, Manuelidis, page 1069, Abstract). In a study using guinea pigs, Manuelidis “demonstrated” that “there is a viremia in experimental [CJD].” Manuelidis isolated the buffy coat (which contained white blood cells) from CJD-infected guinea pigs, injected the buffy coat into disease free guinea pigs, and observed the animals for the progression of the disease. Manuelidis theorized that maximal infectivity should reside in the buffy coat rather than in the serum or red blood cells. While it may be true that Manuelidis established that the disease-causing agent is present in the buffy coat of the blood, this information is of a generic kind when compared with the specificity of the present invention (specifically, methods of identifying TSE-infected B-cells and T-

cells in a test sample by collecting the cell types and directly testing them for the presence of prion associated with the TSE). In fact, Manuelidis does not discuss at all the role played by the B-cells and T-cells in transmission of the TSE.

Having recognized that Kuroda and Manuelidis incorrectly taught that the disease-causing agent was a virus, instead of a prion, the Examiner dismissed the importance of this crucial difference by arguing that when the references were published, the prion protein theory of disease was not generally accepted. However, Appellants respectfully submit that it is immaterial for an obviousness rejection whether or not a correct theory of the disease was generally accepted at the time a reference is published. Rather, what is material is whether the reference by itself or in combination with other references renders the invention obvious to a person of ordinary skill in the art (hereinafter referred to as a "skilled artisan"). Applicants contend that given that Kuroda and Manuelidis were fundamentally wrong about the nature of the disease-causing agent and were not even aware of the existence of prions, the references simply cannot render obvious the method involving the steps of collecting B-cells and/or T-cells from a test sample and directly testing these cell types for the presence of prions associated with TSE.

According to the Examiner, "even if the references erroneously referred to the disease-causing agent as a virus this (sic) does not detract from the important observation made in the references" (See, Office Action mailed on September 21, 2005, page 4). The "important observation" is presumably that CJD is associated with

lymphocyte cells. While it is true that the references disclosed that the highest concentration of the “virus” was in lymphocyte cells, it is unclear how this makes it obvious to test specifically B-cells and T-cells for the presence of prion associated with TSE. The Examiner argues that it would have been obvious at the time the invention was made to improve the sensitivity of antibody-based assays by collecting samples containing B-cells and/or T-cells and testing for the presence of TSE using an antibody-based system. However, the Examiner cannot have it both ways. On the one hand, the Examiner claims that a skilled artisan would disregard the scientific conclusion of Kuroda and Manuelidis that CJD is a virus-based infection. On the other hand, the Examiner claims that the same skilled artisan would accept Kuroda and Manuelidis’ conclusions that lymphocyte cells are associated with CJD. The Examiner offers no scientific rationale as to why a skilled artisan would disregard one finding of the references while embracing the another. Applicants submit that it is more likely that a skilled artisan would either agree with Kuroda and Manuelidis that CJD is a viral infection (and in that case, would not be motivated to test for abnormal prions) or disregard the references completely (and in that case, would not be motivated to concentrate lymphocyte cells).

Therefore, Applicants respectively submit that the claimed invention is not *prima facie* obvious to a skilled artisan.

B. There Is No Motivation to Combine the References

The Federal Circuit has repeatedly held that three basic criteria must be met to establish a *prima facie* case of obviousness. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine the reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not be based on applicant's disclosure. *Manuel of Patent Examining Procedure* §2142 (8th Edition, October 2005 Revision) citing *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). See also, *In re Jones*, 958 F.2d 347, 21 USPQ2d 1941, 1943 (Fed. Cir. 1992).

In the present application, the Examiner has failed to convincingly demonstrate either a suggestion or motivation to combine the asserted references. The Examiner merely combined the disclosures of Kuroda and Manuelidis demonstrating that B-cells and T-cells (among other lymphocytes) may contain the TSE-causing agent with the disclosures of O'Rourke and Korth's of antibody-based methods of detecting TSEs and asserted that such combination would increase the sensitivity of detection assays. As discussed above, Kuroda and Manuelidis mistakenly identified a virus as a TSE-causing agent and indicated that injection of lymphocytes into healthy animals leads to the TSE infection. Neither Kuroda nor Manuelidis taught or suggested that prions played a crucial role in the transmission of TSEs. Korth taught a monoclonal antibody specific for

PrP^{Sc} in brain homogenates and O'Rourke taught antibody-based methods to detect PrP^{Sc} as an indication of TSEs.

More specifically, O'Rourke described detection of PrP^{Sc} using antibodies that bind to PrP^{Sc}. While O'Rourke focused on third eyelid lymphoid tissues in ruminant animals, nowhere did O'Rourke disclose or suggest the specific role played by B-cells in transmission of TSEs.

The Examiner's rationale is as follows:

Both O'Rourke et al. and Korth et al. teach methods of detecting the disease form of prion protein after proteinase K digestion followed by SDS-page electrophoresis and blotting onto a membrane. One of ordinary skill in the art would have a high expectation of success in applying the techniques taught by O'Rourke et al. or Korth et al. to the infected tissue disclosed by Kuroda et al. or Manuelidis et al. It would have been obvious at the time the invention was made to improve the sensitivity of the TSE tests by collecting samples containing B cells and/or T cells and testing for the presence of TSE using an antibody based system. The ordinary artisan at the time the invention was made would have been motivated to this (sic) in order to avoid having to utilize animals in order to test for infectivity in the B and/or T cell population. The ordinary artisan at the time the invention was made would have reasonably expected that concentrating a cell type known to be infected with the TSE agent would increase the sensitivity of detection assays, including antibody-based assays. In addition, it was well known in the art at the time the invention was made that once an antibody was developed, the antibody could be used with a reasonable expectation of success to detect an antigen on intact cells, as in a buffy coat of whole blood, by either mounting them on slides for immunohistochemical analysis; or by using other techniques well known in the art at the time the invention was made for intact cell analysis with antibodies. Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references (Office Action mailed on November 14, 2003, pages 6-7).

It appears that the Examiner's rationale for combining the references is that a skilled artisan would have been motivated to do so to avoid having to utilize live animals to test for TSE infectivity. According to the Examiner, the skilled artisan would have concluded that B-cells and T-cells are known to be infected with the TSE-causing agent and would have been motivated to concentrate these cell types to increase the sensitivity of detection assays.

However, this reasoning is flawed. According to the Federal Circuit, the motivation or suggestion to combine the references must be found either in the references themselves, in the knowledge generally available to one of ordinary skill in the art, or from the nature of the problem to be solved, leading the inventors to look to the references for possible solutions to that problem. *Ruiz v. A.B. Chance Co.*, 69 USPQ2d 1686, 1691 (Fed. Cir. 2004). Both the suggestion and the reasonable expectation of success must be found in the prior art, not in the applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). Moreover, the showing of a motivation to combine must be clear and particular, and it must be supported by actual evidence. *Teleflex Inc. v. Ficosa North America Corp.*, 63 USPQ2d 1374, 1387 (Fed. Cir. 2002).

It is beyond dispute that neither Kuroda nor Manuelidis contain any hints or suggestions to combine their teachings with an antibody-based methods to detect abnormal prions associated with TSEs. Indeed, it would be impossible for either Kuroda or Manuelidis to contain such hints or suggestions given that both of these references

mistakenly believed that TSEs were caused by a virus. The knowledge generally available to a skilled artisan also does not contain any motivation or suggestion to combine the references. By 1997 (the priority date of the instant application), the majority of scientific literature would have led a skilled artisan to heavily discount the disclosures of Kuroda and Manuelidis since by then the emerging scientific consensus was that the infective agent of TSE was not a virus. In fact, both O'Rourke and Korth identify prions, not viruses, as TSE-causing agents. Why would a skilled artisan be motivated to combine the references which identify different disease-causing agents? Finally, the nature of the problem to be solved would not lead a skilled artisan to combine the references. The problem to be solved was to develop a reliable method of identifying and/or monitoring TSE in infected organisms or in organisms suspected of being infected. There was no motivation to combine the references which taught that TSE was caused by a virus with references which taught the antibodies to abnormal prions and antibody-based methods to detect abnormal prions.

It is true that the recognition that some advantage or expected beneficial result would have been produced by combining references provides the strongest rationale for their combination. *In re Sernaker*, 702 F.2d 989, 994-95, 217 USPQ 1, 5-6 (Fed. Cir. 1983). However, in the instant application, a skilled artisan would not have expected any advantage or beneficial result from the combination of the references since a skilled artisan who believed in the prion nature of TSE disease would have discounted the findings of researchers who believed that TSEs were caused by a virus.

The Federal Circuit has consistently cautioned Examiners against succumbing to a hindsight-based analysis. “Our case law makes clear that the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references.” *In re Dembiczak*, 175 F.3d 994, 999 (Fed. Cir. 1999). “Combining prior art references without evidence of such a suggestion, teaching or motivation simply takes the inventor’s disclosure as a blueprint for piecing together the prior art to defeat patentability—the essence of hindsight.” *Id.*

Of course, in hindsight it is “obvious” to combine the disclosures of Kuroda, Manuelidis, Korth, and O’Rourke to develop a reliable test for TSEs. However, it is “obvious” only after Applicants’ careful and meticulous investigation of the role that B-cells play in transmission of TSEs. Prior to the Applicants’ invention, the roles of different components of the immune system, and in particular, the primary role of B-cells in transmission of TSEs were not known. Applicants were the first to identify B-cells and B-cells dependent processes as a limiting factor in the development of TSE after peripheral infection (See, Specification, page 27, first paragraph). Even if one assumes that Kuroda and Manuelidis determined that lymphocytes were involved in transmission of TSEs, neither Kuroda nor Manuelidis have identified B-cells as the specific subset of the lymphoreticular system (hereinafter “LRS”) responsible for disease spread. Without this knowledge, it cannot be said that it was obvious to detect the presence of TSE-infected B-cells and/or T-cells by contacting the proteinase K-digested cells with an anti-PrP antibody.

Accordingly, there is no motivation or suggestion to combine the references.

C. Korth Reference Teaches Away From Applicants' Invention

A fair reading of the Korth reference would likely teach away from Applicants' invention. A prior art reference may be considered to teach away when "a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant." *In re Gurley*, 27 F. 3d 551, 553 (Fed. Cir. 1994).

While the Examiner claims that the Korth reference supports his determination of obviousness, "it is impermissible within the framework of 35 U.S.C. §103 to pick and choose from any one reference only so much of it as will support a given position to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one skilled in the art." *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.*, 796 F.2d 443 (Fed. Cir. 1986). Korth claimed to have identified the antibody, 15B3, specific for an abnormal prion, PrP^{Sc}. This antibody specifically precipitates bovine, murine or human PrP^{Sc}, but not PrP^C (See, Korth, page 74). According to Korth, "the identification of an antibody that binds selectively to PrP^{Sc} from various species provides a new means to identify PrP^{Sc} directly **without using proteinase K digestion as a criterion**" (emphasis by Applicants) (See, Korth, page 77). In other words, Korth suggests that a skilled artisan would be able to avoid the step of proteinase K digestion and to precipitate PrP^{Sc} directly. However, Applicants' claims

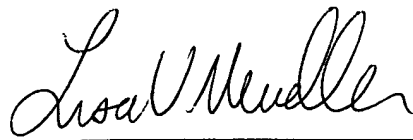
specifically recite a step of subjecting B-cells and/or T-cells to proteinase K digestion (See, claims 35-37). It is not surprising since Applicants' method involves using an antibody which is not specific for PrP^{Sc} and therefore, requires a step of proteinase K digestion to allow the antibody to preferentially bind to proteinase K-resistant PrP^{Sc}.

Therefore, a fair reading of the Korth reference would not have led a skilled artisan to Applicants invention and, in fact, would have led a skilled artisan in a different direction.

CONCLUSION

In conclusion, the none of the cited references render the claimed invention obvious. Neither Kuroda nor Manuelidis teach that B-cells and/or T-cells can transmit TSE. While the references teach that lymphocyte fractions (Kuroda) and buffy coat (Manuelidis) may play a role in TSE, neither of these references focused specifically on B-cells and/or T-cells. Moreover, both Kuroda and Manuelidis mistakenly believed that CJD was caused by a virus and were not even aware of the existence of prions, the true pathogenic agents causing TSE. O'Rourke and Korth do not cure these deficiencies since neither O'Rourke nor Korth disclose or suggest the specific role played by B-cells and/or T-cells in transmission of TSEs. Furthermore, there is no motivation or suggestion to combine these references to arrive at a reliable test for TSE, such as the test claimed in the instant invention. Accordingly, Applicants respectfully submit that the rejection of claims 35-37 under 35 U.S.C. §103 (a) as being unpatentable over O'Rourke, and/or Korth in view of Kuroda and/or Manuelidis is in error and should be reversed.

Respectfully submitted,

By 

Lisa V. Mueller, Reg. No. 38,978

Wood, Phillips, Katz,
Clark & Mortimer
500 West Madison Street
Suite 3800
Chicago, Illinois 60661-2511
Tel: (312) 876-2109
Fax: (312) 876-2020

Claims Appendix

35. A method of identifying TSE-infected B-cells associated with transmissible spongiform encephalopathy in a test sample, the method comprising the steps of:
obtaining a test sample suspected of TSE infection;
collecting B-cells from the test sample;
subjecting said B-cells to homogenization;
subjecting said homogenized B-cells to proteinase K digestion;
subjecting said digested B-cells to SDS Page immunoaffinity chromatography

blots;

contacting said blots with an anti-PrP antibody,
wherein the presence of a signal from said anti-PrP antibody-PrP complex in the sample is indicative of TSE-infected B-cells;
identifying TSE-infected B-cells based on the presence of said signal; and
wherein the identification of TSE-infected B-cells is associated with TSE promulgation and primary infection.

36. A method of identifying TSE-infected T-cells associated with transmissible spongiform encephalopathy in a test sample, the method comprising the steps of:

obtaining a test sample suspected of TSE infection;
collecting T-cells from the test sample;
subjecting said T-cells to homogenization;
subjecting said homogenized T-cells to proteinase K digestion;
subjecting said digested T-cells to SDS Page immunoaffinity chromatography

blots;

contacting said blots with an anti-PrP antibody,
wherein the presence of a signal from said anti-PrP antibody-PrP complex in the sample is indicative of TSE-infected T-cells;
identifying TSE-infected T-cells based on the presence of said signal; and
wherein the identification of TSE-infected T-cells is associated with TSE promulgation and secondary infection.

37. A method of identifying TSE-infected B-cells and TSE-infected T-cells associated with transmissible spongiform encephalopathy in a test sample, the method comprising the steps of:

obtaining a test sample suspected of TSE infection;
collecting B-cells and T-cells from the test sample;
subjecting said B-cells and T-cells to homogenization;
subjecting said homogenized B-cells and T-cells to proteinase K digestion;
subjecting said digested B-cells and T-cells to SDS Page immunoaffinity chromatography blots;
contacting said blots with an anti-PrP antibody,
wherein the presence of a signal from said anti-PrP antibody-PrP complex in the sample is indicative of TSE-infected B-cells and TSE-infected T-cells;

identifying TSE-infected B-cells and TSE-infected T-cells based on the presence of said signal; and
wherein the identification of TSE-infected B-cells is associated with TSE promulgation and primary infection and the identification of TSE-infected T-cells is associated with TSE promulgation and secondary infection.

Evidence Appendix

Copies of cited references are enclosed.

Related Proceedings Appendix

Not Applicable.

surface of the antennal lobes through a small window cut in the head cuticle; these experiments were done without the experimenter knowing whether the drop contained PCT or saline. In the second method (group 2, Table 1), 0.1 nl saline or picrotoxin (100 μ M–1 mM in saline) was injected directly into the antennal lobes through a small window in the head just above the base of each antenna using a Picospritzer (General Valve)²⁴. Injections gave the same results as topical applications, although PE response rates were reduced, as commonly observed after extensive surgery. After a time t_1 (10, 45, 60 or 90 min) for recovery, animals were trained by using the following protocol^{14,24}: 6 paired presentations of odorant (4-s pulse into a vented air stream) and sucrose (0.4 μ l of 1.25 M solution for group 1, 2 μ l of 2 M solution for group 2, presented to the antenna and the proboscis 3 s after odorant pulse onset), every 2 min (group 1) or 30 s (group 2). Animals showing a PE response in each trial were selected to receive 2 or 3 extinction (odour only) trials (one with each of the 2 or 3 test odours; see below) 90 min (group 1) or 60 min (group 2) after conditioning. The odorants used for conditioning were 1-hexanol or 1-octanol. Groups were counterbalanced to contain roughly equal numbers of bees trained with either alcohol. The odours used for testing (1-octanol, 1-hexanol, geraniol) were presented to each animal in a randomized order. Generalization between the alcohols and geraniol is typically low²⁵. We used the percentage of subjects that responded to an extinction test as the response measure. Results were compared with χ^2 statistics because behavioural data were categorical (PE or no PE). Statistical values are one-tailed because generalization responses were not expected to exceed the response levels to conditioned stimuli.

Received 9 July; accepted 6 August 1997.

- Adrian, E. D. Olfactory reactions in the brain of the honeybee. *J. Physiol. (Lond.)* 100, 459–473 (1942).
- Gray, C. M. & Skinner, J. E. Centrifugal regulation of neuronal activity in the olfactory bulb of the waking rabbit as revealed by reversible cyrogenic blockade. *Exp. Brain Res.* 69, 378–386 (1988).
- Gelper, A. & Tuck, D. W. Odour-mediated collective network oscillations of olfactory interneurons in a terrestrial mollusc. *Nature* 345, 437–440 (1990).
- Delaney, K. R. et al. Waves and stimulus-modulated dynamics in an oscillating olfactory network. *Proc. Natl Acad. Sci. USA* 91, 669–674 (1994).
- Laurent, G. & Davidowitz, H. Encoding of olfactory information with oscillating neural assemblies. *Science* 263, 1872–1875 (1994).
- Gray, C. M. & Singer, W. Stimulus specific neuronal oscillations in orientation columns of cat visual cortex. *Proc. Natl Acad. Sci. USA* 85, 1698–1702 (1988).
- Shige, W. & Gray, C. M. Visual feature integration and the temporal correlation hypothesis. *Annu. Rev. Neurosci.* 10, 535–586 (1995).
- Neuechwander, S. & Vardi, E. J. Visually triggered neuronal oscillations in the pigeon—an accommodation study of neural activity. *Eur. J. Neurosci.* 7, 870–881 (1995).
- Laurent, G., Wehr, M. & Davidowitz, H. Temporal representations of odors in an olfactory network. *J. Neurosci.* 16, 3837–3847 (1996).
- Wehr, M. & Laurent, G. Temporal combinatorial encoding of odours with oscillations. *Nature* 384, 162–166 (1996).
- Laurent, G. Dynamical representation of odors by oscillating and evolving neural assemblies. *Trends Neurosci.* 19, 489–496 (1996).
- MacLeod, K. & Laurent, G. Distinct mechanisms for synchronization and temporal patterning of odor-encoding neural assemblies. *Science* 274, 976–979 (1996).
- Kuwahara, M. Bildung des bedingten reflexes von proboscis typen bei der honigbiene, *Apis mellifica*. *J. Fac. Sci. Hokkaido Univ. (Ser. VI Zool.)* 13, 458–464 (1957).
- Bitterman, M. E., Menzel, R., Fietz, A. & Schilke, S. Classical conditioning of proboscis extension in honeybees (*Apis mellifica*). *J. Comp. Psychol.* 97, 107–119 (1983).
- Menzel, R. & Bitterman, M. E. In *Neurobiology and Behavioral Physiology* (eds Huber, R. & Muel, H.) 205–215 (Springer, New York, 1983).
- Smith, B. H. & Menzel, R. The use of electrocytogram recordings to quantify odour discrimination in the honey bee, *Apis mellifica*. *J. Insect Physiol.* 35, 569–575 (1989).
- Menzel, R. In *Neurobiology of Comparative Cognition* (eds Keesen, R. P. & Olson, D. S.) 257–292 (Edinburgh, New Jersey, 1990).
- Menzel, R., Mischke, B., Böcker, P. & Sugawara, M. In *Modulation of Synaptic Plasticity in Nervous Systems* NATO ASI series, Vol. H19 (eds Hartung, G. & Spatz, H.-C.) 333–350 (Springer, Berlin, 1988).
- Yokoi, M., Mori, K. & Nakazawa, S. Reinforcement of odor molecule tuning by dendrodendritic synaptic inhibition in the olfactory bulb. *Proc. Natl Acad. Sci. USA* 92, 3371–3375 (1995).
- Jörges, J., Rattner, A., Gheis, C. G. & Menzel, R. Representation of odours and odour mixtures visualized in the honeybee brain. *Nature* 387, 283–288 (1997).
- Murphy, V. N. & Pitz, P. E. Coherent 25 Hz to 35 Hz oscillations in the sensorimotor cortex of awake behaving monkeys. *Proc. Natl Acad. Sci. USA* 89, 5670–5674 (1992).
- Gray, C. M. Synchronous oscillations in neuronal systems: mechanisms and functions. *J. Comput. Neurosci.* 1, 11–38 (1994).
- Mitschke, J. Neural correlates of olfactory learning-paradigms in an identified neuron in the honeybee brain. *J. Neurophysiol.* 69, 609–623 (1993).
- Bhagavan, S. & Smith, B. H. Olfactory conditioning in the honey-bee, *Apis mellifica*—effects of odor intensity. *Physiol. Behav.* 61, 107–117 (1997).
- Smith, B. H. An analysis of blocking in binary odour mixtures: An increase but not a decrease in intensity of reinforcement produces unblocking. *Behav. Neurosci.* 111, 57–69 (1997).
- Macmillan, C. S. & Menzel, R. An investigation of the role of dopamine in the antennal lobes of the honeybee, *Apis mellifica*. *J. Comp. Physiol.* A 160, 359–366 (1987).

Acknowledgements. We thank K. MacLeod, L. Kay, M. Wehr, A. Herberichs and H. Krapp for their helpful comments. Supported by an NSRF (NIDCD) (Schwartz O.S.), an NIH grant (B.H.S.), an NSF grant, an NSF Presidential Faculty Award, and a grant from the Sloan Center for Theoretical Neuroscience at Caltech (G.L.).

Correspondence and requests for materials should be addressed to G.L. (e-mail: laurent@caltech.edu).

Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody

C. Korth*, B. Stierli†, P. Strelti, M. Moser*, O. Schaller*, R. Fischer†, W. Schulz-Schaeffer†, H. Kretzschmar†, A. Raeber†, U. Braun†, F. Ehrensperger†, S. Hornemann†, R. Glockshuber†, R. Rieks, M. Billeter†, K. Wüthrich† & B. Oesch*

* Prionics AG, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

† Brain Research Institute, University of Zürich, 8029 Zürich, Switzerland

‡ Institut für Biochemie, ETH Zürich, 8092 Zürich, Switzerland

§ Institut für Neuropathologie, Universität Göttingen, 37075 Göttingen, Germany

|| Institut für Neuropathologie, University of Zürich, 8091 Zürich, Switzerland

¶ Klinik für Wiederkäufer- und Pferdemedizin, University of Zürich, 8057 Zürich, Switzerland

‡ Institut für Veterinärpathologie, University of Zürich, 8057 Zürich, Switzerland

§ Institut für Molekularbiologie und Biophysik, ETH Zürich, 8093 Zürich, Switzerland

Prions are infectious particles causing transmissible spongiform encephalopathies (TSEs). They consist, at least in part, of an isoform (PrP^{Sc}) of the ubiquitous cellular prion protein (PrP^C). Conformational differences between PrP^C and PrP^{Sc} are evident from increased β -sheet content and protease resistance in PrP^{Sc} (refs 1–3). Here we describe a monoclonal antibody, 15B3, that can discriminate between the normal and disease-specific forms of PrP. Such an antibody has been long sought as it should be invaluable for characterizing the infectious particle as well as for diagnosis of TSEs such as bovine spongiform encephalopathy (BSE) or Creutzfeldt-Jakob disease (CJD) in humans. 15B3 specifically precipitates bovine, murine or human PrP^{Sc}, but not PrP^C, suggesting that it recognizes an epitope common to prions from different species. Using immobilized synthetic peptides, we mapped three polypeptide segments in PrP as the 15B3 epitope. In the NMR structure of recombinant mouse PrP, segments 2 and 3 of the 15B3 epitope are near neighbours in space, and segment 1 is located in a different part of the molecule. We discuss models for the PrP^{Sc}-specific epitope that ensure close spatial proximity of all three 15B3 segments, either by intermolecular contacts in oligomeric forms of the prion protein or by intramolecular rearrangement.

PrP-null mice were immunized with full-length recombinant bovine PrP. After fusion of spleen cells with myeloma cells, we selected ~50 hybridoma cells that produced monoclonal antibodies recognizing either native bovine PrP^{Sc} (PrP^{BSE}) immobilized on nitrocellulose or recombinant bovine PrP (rbPrP) in an enzyme-linked immunosorbent assay (ELISA). One of these antibodies (15B3) was selected for binding to protease-digested BSE brain homogenates; a second (6H4) efficiently recognized recombinant PrP. On western blots, 6H4 recognized rbPrP, as well as bovine, human, mouse and sheep PrP^C, whereas 15B3 did not react with any form of PrP (results not shown). To determine the reactivity of these antibodies with native PrP^C and PrP^{Sc}, we immunoprecipitated PrP from brain homogenates of normal and BSE-infected cattle. The precipitated proteins were then analysed on western blots using a rabbit polyclonal antiserum to rbPrP (Fig. 1). The 6H4 antibody precipitated PrP from BSE as well as from normal brain homogenates; 15B3 precipitated only PrP from brain homogenates of BSE-diagnosed cattle (Fig. 1a). Upon proteinase K treatment, normal PrP is completely digested, whereas the 33K–35K form of PrP^{Sc} is shortened to 27K–30K (PrP 27–30), probably as a result of

degradation of the amino-terminal segment of residues 23–90, analogous to hamster PrP^{Sc} (ref. 3). Digestions of brain homogenates or immunoprecipitates with proteinase K are shown in Fig. 1b. Proteinase K digestion of BSE homogenates or the immunoprecipitate with 15B3 yields the PrP^{Sc}-specific band of 27K–30K (Fig. 1b). Not all of the immunoprecipitated PrP was protease-resistant, suggesting that 15B3 recognizes multiple forms of disease-specific PrP with different sensitivities to proteinase K. Apparently, PrP with properties characteristic for PrP^{Sc} but without protease resistance occurs as an intermediate in the generation of fully proteinase-resistant PrP^{Sc} (ref. 4). No PrP 27–30 was found in normal homogenates or immunoprecipitates with protein A only (Fig. 1b) or 6H4 (not shown). 15B3 therefore seems to be a PrP^{Sc}-specific antibody, even though we immunized with recombinant bovine PrP. Injection of rPrP into Tg20 mice overexpressing mouse PrP^{Sc} has not produced disease for 430 days, whereas two out of four mice injected with a homogenate from the medulla of a BSE-affected cow have come down with TSE at 388 and 426 days (A.R., C.K. and B.O., unpublished results). In addition, it has been reported that recombinant PrP is not infectious⁵. Recombinant PrP is also not protease-resistant, which is a hallmark of PrP^{Sc} (C.K., unpublished observation)⁶. The model of Lansbury and Caughey⁷, which postulates that the two isoforms of PrP are in a dynamic equilibrium, provides a possible explanation for these findings. By immunizing with large amounts of normal PrP, a small portion of the protein might, according to this hypothesis, have been in the scrapie-specific conformation when triggering the immune response. Alternatively, recombinant PrP molecules might transi-

ently associate (see below and Fig. 3b), and thereby form the prion-specific epitope when acting as an immunogen.

We further analysed the species specificity of 15B3 using mouse scrapie-infected brain homogenates (Fig. 1c) and brain homogenates from CJD type-1 patients (Fig. 1d)¹⁰. For comparison, the mouse brain homogenates of PrP-null as well as normal and scrapie-infected wild-type mice, and the immunoprecipitates corresponding to twice the amount of the homogenates are shown (Fig. 1c). Mouse PrP^{Sc} could be efficiently precipitated by 15B3, as indicated by the presence of PrP 27–30 after the digestion with proteinase K (Fig. 1c, lane a). When brain homogenate was treated with proteinase K before the precipitation, 15B3 was also able to precipitate PrP 27–30 (Fig. 1c, lane b), indicating that the N-terminal segment 23–90 is not critical for binding of 15B3 to PrP^{Sc}, even though precipitation of intact PrP^{Sc} appeared to be more efficient than that of PrP 27–30. Proteinase K digestion causes the formation of large aggregates (scrapie-associated fibrils) which may mask the 15B3 epitope. Surprisingly, 15B3 also specifically recognized PrP^{CJD} from sporadic CJD cases but not human PrP^C (Fig. 1d), even though the amino-acid sequence in the regions of the 15B3 epitope is not fully conserved (see below, and Fig. 2b).

The epitopes recognized by the two antibodies were determined by using a gridded array of synthetic peptides consisting of 104 13-residue peptides sequentially shifted in steps of two amino acids and covering the whole mature bovine PrP sequence. A single linear epitope (DYEDRYRE; corresponding to positions 144–152 of human PrP¹¹) was mapped for 6H4, whereas three distinct peptide sequences were found to form the 15B3 epitope (amino acids

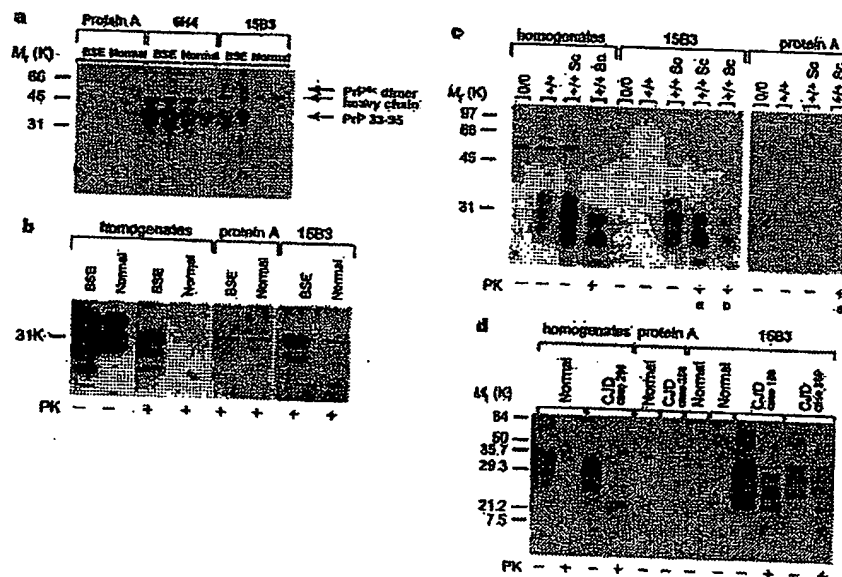


Figure 1 Immunoprecipitation of bovine, mouse and human PrP with monoclonal antibodies 15B3 and 6H4. **a**, The supernatant of a centrifuged homogenate from the medulla of two different BSE-diagnosed or two normal animals was incubated with antibodies 6H4 or 15B3. Antibodies were precipitated with protein A (15B3) or protein G-agarose (6H4). As a control, protein A only was incubated without antibodies. Precipitates were analysed on a western blot for the presence of PrP using a polyclonal rabbit antiserum to bovine PrP and goat anti-rabbit Ig coupled to alkaline phosphatase. Signals were developed with chemiluminescence substrates. Crossreaction of the secondary antibody with immunoprecipitated mouse immunoglobulins leads to the prominent band at about 50K. Note the 60K band characteristic for PrP^{Sc} in the 15B3 but not in the 6H4 immunoprecipitations⁸. **b**, Proteinase K digestion of PrP^{Sc} immunoprecipitated with mAb 15B3. Undigested and digested bovine brain homogenates were compared

to proteinase K digested immunoprecipitates with protein A-agarose only or with 15B3. The sharp band at 31K represents a crossreactivity of the secondary antibody with proteinase K. The same immunoprecipitates and method of analysis were used as in **a**. **c**, Immunoprecipitation of mouse PrP^{Sc} with mAb 15B3. Homogenates from PrP-null mice (0/0) or wild-type mice (normal (+/+)) or scrapie-infected (+/+Sc) were immunoprecipitated with mAb 15B3 or protein A-agarose only and analysed by western blotting as described. Digestion with proteinase K after (a) or before (b) the immunoprecipitation is indicated. Detection of PrP was done as described. **d**, Immunoprecipitation of human PrP^{CJD} with mAb 15B3. Brain homogenates (cerebellum) from normal persons or CJD patients type 1 (ref. 10) were immunoprecipitated and analysed as described for **a**. Two representative examples from a total of 4 normal persons and 4 CJD cases are shown.

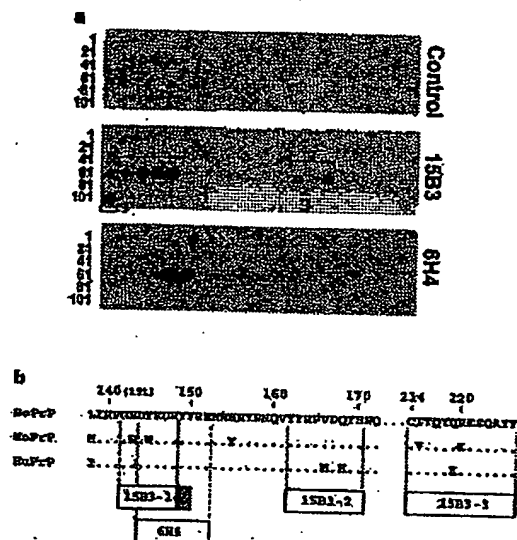


Figure 2 Determination of epitopes for mAbs 15B3 and 6H4. A gridded array of synthetic peptides corresponding to bovine PrP was incubated with 15B3, 6H4 or with secondary antibody only (peroxidase-labelled goat anti-mouse Ig; control). Bound antibody was visualized with chemiluminescence. Each spot corresponds to a 13-amino-acid peptide, which is shifted by two amino acids along the bovine PrP sequence relative to the previous peptide. Peptides were covalently attached at the C-terminus to a cellulose support. A total of 104 peptides were used to cover the whole bovine PrP sequence including the six octapeptide repeat sequences²¹.

b. Minimal sequences recognized by 15B3 or 6H4 antibodies in the array of synthetic bovine PrP peptides. The polypeptide segments of the 15B3 epitope are numbered as they occur in the amino acid sequence. The first 15B3 segment extends by two amino acids C-terminally (grey box) if spot number 62, which binds 15B3 only weakly, is excluded. The numbering of the sequence is according to human PrP^{Sc}; the number in brackets indicates the position in the bovine PrP sequences used for the construction of the array of synthetic peptides. Differences with the human and mouse PrP sequences are indicated.

142-148, 162-170, and 214-226; Fig. 2a, b). The relative positions of these partial epitopes in the amino-acid sequence revealed an overlap of the 6H4 epitope with the first segment of the 15B3 epitope (Fig. 2b).

Mapping of the 15B3 epitope onto the NMR structure of the C-terminal domain of mouse PrP (ref. 12) reveals close proximity of the peptide segments 2 and 3, but a much larger spatial separation of the segment 1 from either of the other two components (Fig. 3a). The peptide segment 1 occupies the N-terminal half of helix 1 plus the two residues preceding it, and it is recognized by 6H4 in PrP^C as well as by 15B3 in PrP^{Sc}. This finding would be compatible with either of the two following assumptions: (1) 15B3 recognizes segment 1 of its epitope only in concert with the segments 2 and 3; (2) the polypeptide segment of helix 1 is differently folded in PrP^C and PrP^{Sc}. Component 2 of the 15B3 epitope is in the loop connecting the second β -strand to the second helix, with two thirds of it in a disordered region in the three-dimensional structure, and component 3 is located at the C-terminal end of helix 3 (refs 12, 13). The peptide segments 2 and 3 are located in the proposed binding region for 'protein X' (ref. 14), which is characterized by significant alterations of the electrostatic surface potential among different mammalian species¹⁵: human PrP differs from bovine and mouse PrP in the replacement of the glutamine residues 168 and 219 by glutamic acid residues, as well as by conservative substitutions at positions 166 and 215. As bovine, mouse and human PrP^{Sc} are all precipitated by 15B3 (Fig. 1a-d), this antibody probably binds to the conserved residues in this region.

A single continuous 15B3 binding site could be formed either by aggregation of two or several PrP molecules¹⁶, or by structural rearrangement of a single PrP molecule, or by a combination thereof. Figure 3b suggests a spatial arrangement of a PrP dimer that would bring all three segments of the 15B3 epitope into spatial proximity, with minimal conformational changes of the individual molecules. It is based on the observation of a structural similarity between PrP(121–231) and haemoglobins, which allows a superposition of the helices 1, 2 and 3 of PrP^C onto the helices 1, 6 and 7 of the haemoglobin β -subunit, with a root-mean-square distance for the polypeptide backbone of 2.4 Å. Superposition of two molecules

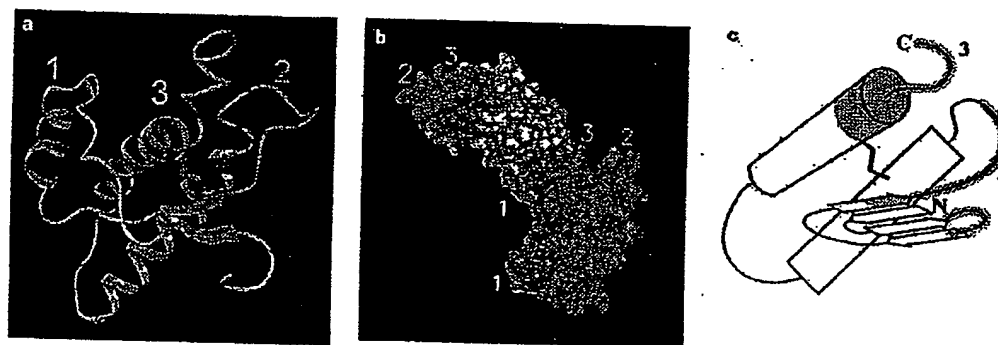


Figure 3 The epitope of the monoclonal antibody 15B3 in the three-dimensional prion protein structure. **a**, Mapping of the 15B3 epitope onto a ribbon drawing of the NMR structure of the C-terminal domain PrP^C(121–231) of mouse PrP^C (refs 12, 26). The three segments of the 15B3 epitope are coloured yellow (1), violet (2) and cyan (3) in the order in which they occur in the amino acid sequence (Fig. 2b). The residual parts of the molecule and the single disulphide bridge are grey. Regular secondary structures are indicated by ribbons for helices and arrows for β -strands. Drawings in **a** and **b** were prepared with MOLMOL²⁷. **b**, Surface representation of two PrP^C(121–231) molecules after superposition onto two β -subunits of the crystal lattice of sickle cell haemoglobin¹⁷ (Protein Data Bank entry

(1HB5). The segments of the 1583 epitope are numbered and coloured as in a. The superposition included the backbone atoms of residues 145–154, 178–189 and 201–217 of the helices 1, 2 and 3 of PrP(121–231) and of residues 5–14, 106–116 and 125–141 of the helices 1, 6 and 7 of haemoglobin S (r.m.s.d. = 2.4 Å). c, Hypothetical fold of the prion protein that would bring all three components of the 1583 epitope into spatial proximity. The two cylinders represent the disulphide-linked helices 2 and 3 in the orientation of a. Helix 1 and the β -sheet have been replaced by four β -strands that form a greek key motif. The chain termini are labelled N and C, and the segments of the 1583 epitope are numbered as in a.

of PrP(121–231) onto two adjacent β -chains in the crystal lattice of haemoglobin S¹⁷ (PDB entry 1HBS) brings the peptide segment 1 of one PrP molecule near to the segments 2 and 3 of the other molecule (Fig. 3b). This superposition aligns residue 6 of the β -chains of sickle cell haemoglobin with Trp at position 145 of the mouse prion protein, which is located in the middle of the epitope segment 1 and is fully exposed to solvent. Mutation of Glu 6 to valine is responsible for the formation of haemoglobin aggregates in sickle cell anaemia.

Intramolecular structural rearrangement bringing all three segments of the 15B3 epitope into close spatial proximity might involve the first helix and lead to an extension of the existing β -sheet¹⁸. In Fig. 3c, the resulting β -structure is assumed to consist of four strands aligned to form a greek-key motif. Other PrP^{Sc} models that would also lead to close approach of the three segments of 15B3 epitope have been described¹⁹.

The identification of an antibody that binds selectively to PrP^{Sc} from various species provides a new means to identify PrP^{Sc} directly without using proteinase K digestion as a criterion. It will be interesting to see whether 15B3 will be able to neutralize infectivity and thus be a potential therapeutic reagent. The low level of PrP^{Sc} in peripheral tissues has made it difficult to use it as a marker for prion diseases¹⁹. Affinity selection of PrP^{Sc} with 15B3 will allow enrichment of the abnormal isoform of PrP and thus lower the detection limit for PrP^{Sc}, so a prion test for living humans or animals is conceivable. The mapping and three-dimensional modelling of the 15B3 epitopes has provided a view of a prion-disease-specific epitope and may represent a starting point for the production of further diagnostic or therapeutic tools for TSEs. □

Methods

Materials. BSE material was from naturally occurring Swiss cases of BSE, CJD brain material from patients suffering from CJD type 1 (ref. 10), which had been diagnosed using histopathology and immunohistochemistry for PrP. For mouse scrapie material, CD-1 mice were experimentally infected with the RML strain²⁰.

Preparation of recombinant bovine PrP. The bovine PrP open reading frame was amplified by PCR from genomic DNA using the primers 5'-GGGAATTC-CATATGAAGAAGGACCAAAACCTG and 5'-CGGGATCCCTATTAACCTG-CGCCCTGGTGGTAA. The resulting PCR product was cloned into pET11a (Novagen) using the *Nde*I and the *Bam*HI restriction sites. The resulting plasmid (pPrP3) was transfected into *E. coli* BL21 (DE3). Bacteria were grown to OD₆₀₀ = 0.8 then induced with 1 mM IPTG and further grown at 30 °C for 3 h. rPrP corresponding to the mature form of bovine PrP containing six octapeptides²¹ was purified from inclusion bodies after solubilization in 8 M urea, 10 mM MOPS/NaOH, pH 7.0 (UM-buffer), on a CM-sepharose column (Pharmacia; UM, 0–0.5 M NaCl gradient) and reverse-phase HPLC (Vydac C₄ column, 0.1% trifluoroacetic acid, 0–60% acetonitrile gradient; C.K. and B.O., unpublished results). Resulting fractions contained either oxidized (elution time, 29 min) or reduced PrP (elution time, 34 min). Usually, CM-sepharose fractions were oxidized with 1 μ M CuSO₄ for 1 h before purification by reverse-phase HPLC. Purified rPrP was analysed by mass spectrometry, indicating a protein of the expected mass in which the N-terminal methionine was uncleaved.

Immunization of PrP-null mice. 100 μ g recombinant bovine PrP in Freund's complete adjuvant was injected into PrP null mice (mixed background 129/Sv and C57BL/6J²²) subcutaneously, and 21 and 42 d later with the same amount of antigen in Freund's incomplete adjuvant. Mice were boosted intraperitoneally (day 48) and intravenously (day 49) with recombinant PrP dissolved in PBS. At day 50, mice were decapitated and splenocytes fused to myeloma cells as described²³. Supernatants of the resulting hybridoma cell lines were screened both by ELISA with recombinant bovine PrP as antigen and by ELISA²⁴ using native, protease-digested brain homogenate of BSE-diseased cattle. Positive hybridoma cells were subcloned three times.

Characterization of antibodies. Supernatants of selected hybridomas were used to probe bovine PrP in brain homogenates or recombinant PrP on western blots. To determine the epitopes, antibodies were incubated with a gridded array of peptides comprising 104 polypeptides of 13 amino acids, shifted by two

amino acids and covering the entire mature bovine PrP sequence containing six octapeptide repeats²¹. The peptides were covalently attached at their C termini to a cellulose support as individual spots (Jettini Biotech, Berlin). Bound antibody was detected with goat anti-mouse immunoglobulin coupled to horseradish peroxidase and chemiluminescence. Signals were recorded on Hyperfilm ECL (Amersham). For immunoprecipitation, 200 μ l 1% brain homogenates (precleared by centrifugation at 13,000g for 15 min) were incubated for 2 h at room temperature with 200 μ l 0.25 mg ml⁻¹ antibody-containing serum-free medium; after incubation with an additional 50 μ l protein A- or protein G-coupled agarose (for 15B3 and 6H4, respectively; Boehringer Mannheim) for 2 h at room temperature, agarose beads were centrifuged at 13,000g for 3 min, and the pellet washed according to the manufacturer. Proteinase K (PK) digestions of immunoprecipitates were done with 20 μ g ml⁻¹ PK (Sigma) for 30 min at 37 °C. Pellets were then boiled in SDS-sample buffer for analysis on western blots. Immunoprecipitated PrP was detected with polyclonal antibodies raised against bovine recombinant PrP in rabbits (R 26) followed by incubation with a goat anti-rabbit immunoglobulin coupled to peroxidase or a goat anti-rabbit IgG coupled to alkaline phosphatase. Bound enzymatic activity was visualized with chemiluminescent substrates (ECL, Amersham, or CSPD, Tropix, respectively).

Received 23 July; accepted 2 October 1997.

1. Pan, K. M. et al. Conversion of α -helices into β -sheets features in the formation of the scrapie prion protein. *Proc. Natl Acad. Sci. USA* 90, 10962–10966 (1993).
2. McKinley, M. P., Bohon, D. C. & Prineas, S. B. A protease-resistant protein is a structural component of the scrapie prion. *Cell* 35, 57–62 (1983).
>
3. Oesch, B. et al. A cellular gene encodes scrapie PrP 27–30 protein. *Cell* 60, 735–746 (1985).
4. Doude, N., Lehmann, S. & Haass, D. A. Identification of intermediate steps in the conversion of a mutant prion protein to a scrapie-like form in cultured cells. *J. Biol. Chem.* 272, 11604–11612 (1997).
5. Fierke, M. et al. Prion protein (PrP) with amino-proline deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J.* 15, 1255–1264 (1996).
6. Madsen, J. et al. High-level expression and characterization of a purified 142-residue polypeptide of the prion protein. *Biochemistry* 35, 5528–5537 (1996).
7. Weiss, S., Rieger, R., Edelhof, P., Fisch, E. & Wenzel, E. L. Recombinant prion protein (PrP 27–30) from *Syria golden hamster* reveals proteinase K sensitivity. *Biochem. Biophys. Res. Commun.* 219, 179–179 (1996).
8. Knebel, K. et al. Molecular properties of complexes formed between the prion protein and synthetic peptides. *J. Mol. Biol.* 270, 574–586 (1997).
9. Lumb, R. T. & Coughlin, B. The chemistry of scrapie infection: implications of the 'ice 9' metaphor. *Chem. Biol.* 2, 1–5 (1995).
10. Purdy, P. et al. Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. *Ann. Neurol.* 39, 767–778 (1996).
11. Schiold, H. M., Da Costa, M., Taylor, L., Cohen, R. E. & Prineas, S. B. Prion protein gene variation among primates. *J. Mol. Biol.* 245, 362–374 (1995).
12. Rich, R. et al. NMR structure of the mouse prion protein domain PrP(121–231). *Nature* 382, 180–182 (1996).
13. Glockshuber, R. et al. Three-dimensional NMR structure of a self-folding domain of the prion protein PrP (121–231). *Trends Biochem. Sci.* 22, 241–242 (1997).
14. Thöni, G. C. et al. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* 83, 79–90 (1995).
15. Billster, M. et al. Prion protein NMR structure and species barrier for prion diseases. *Proc. Natl Acad. Sci. USA* 94, 7281–7285 (1997).
16. Wüthrich, J. & Günter, P. A model for prion protein dimerization based on alpha-helical packing. *Biochem. Biophys. Res. Commun.* 226, 777–782 (1996).
17. Podlan, E. A. & Love, W. E. Refined crystal structure of deoxyhaemoglobin S. II. Molecular interactions in the crystal. *J. Biol. Chem.* 260, 8280–8291 (1985).
18. Huang, Z., Prineas, S. B. & Cohen, R. E. Scrapie prions: a three-dimensional model of an infectious fragment. *Fold Design* 1, 13–19 (1996).
19. Kilmartin, T., Mohr, S. & Thöni, G. Oxygen distribution of proteinase-resistant prion protein in humans and mice with Creutzfeldt-Jakob disease. *J. Gen. Virol.* 70, 3371–3379 (1989).
20. Chomley, R. L. Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet* i, 1378–1379 (1961).
21. Goldmann, W., Hunter, N., Martin, T., Dawson, M. & Hope, J. Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon. *J. Gen. Virol.* 72, 201–204 (1991).
22. Budde, H. et al. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* 356, 577–582 (1992).
23. Kennett, R. H. *Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analysis* (eds Kennett, R. H., McKelvey, T. J. & Beutels, K. B.) 365–367 (Plenum, New York, 1980).
24. Oesch, B., Jansen, M., Nilsson, P. & Fogh, J. Properties of the scrapie prion protein: quantitative analysis of protease resistance. *Biochemistry* 33, 5926–5931 (1994).
25. Priola, S. A., Coughlin, B., Wetzel, K. & Chesebro, B. A 60-kDa prion protein (PrP) with properties of both the normal and scrapie-associated forms of PrP. *J. Biol. Chem.* 270, 3299–3305 (1995).
26. Bick, R., Hornemann, S., Wüthrich, G., Glockshuber, R. & Wüthrich, K. NMR characterization of the full-length recombinant murine prion protein, mPrP(23–231). *FEBS Lett.* 413, 282–288 (1997).
27. Koradi, R., Billeter, M. & Wüthrich, K. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* 14, 51–55 (1996).

Acknowledgements. We thank C. Weissmann for discussion and for PrP null mice, and M. Schaub and his group (Brain Research Institute) for support and encouragement at an early stage of this project. This work was supported by grants from the Schweizerische Nationalfonds to B.O. (SPP Biotechnologie), K.W. and R.G., from the Herman Herzog-Foundation, Basel, to R.G., and a fellowship from the Ciba Foundation to M.M.

Correspondence and requests for materials should be addressed to C.K. (e-mail: ckorth@bio.unizh.ch) or B.O. (e-mail: oesch@bio.unizh.ch).

Creutzfeldt-Jakob Disease in Mice: Persistent Viremia and Preferential Replication of Virus in Low-Density Lymphocytes

YASUO KURODA,[†] CLARENCE J. GIBBS, JR.,* HERBERT L. AMYX, AND D. CARLETON GAJDUSEK

Laboratory of Central Nervous System Studies, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

Received 16 November 1982/Accepted 7 April 1983

The mode of replication of the "unconventional virus" of Creutzfeldt-Jakob disease was studied in BALB/c mice infected intracerebrally. Virus was detected in the brain, spleen, lung, thymus, liver, kidney, and blood, but not in urine, at various time intervals after inoculation. The highest infectivity was present in the spleen from the second through the ninth weeks postinfection. Density gradient separation of spleen cells with colloidal silica (Percoll) revealed that the highest concentration of virus was present in blastoid cells from lower-density (1.05 to 1.07 g/ml) fractions. These results suggest that blastoid cells play an important role as the initial replication site of virus in the pathogenesis of Creutzfeldt-Jakob disease in mice.

It is now well established that Creutzfeldt-Jakob disease (CJD) is an "unconventional" virus-induced slow infection of the central nervous system (CNS) of humans and is classified as one of the naturally occurring "subacute spongiform virus encephalopathies" (9). The pathology of these encephalopathies is not associated with any evidence of host reaction of an inflammatory or immunological type, and virus-specific antibodies have never been detected.

Although structural changes are limited to the CNS, virus can be recovered not only from CNS but also from several extraneural tissues (1, 6, 13, 21, 22, 24, 28). For example, in experimental animals, the virus was present in the CNS, spleen, lymph node, thymus, kidney, liver, cerebrospinal fluid, and peripheral blood (1, 13, 28). The study reported here was undertaken to obtain data on the mode of replication and temporal distribution of CJD virus in relation to onset of the disease and to determine the important implication of the hematogenous route of virus dissemination.

MATERIALS AND METHODS

Mice. Weanling female BALB/c mice obtained from the animal colony of the Frederick Cancer Research Center, Frederick, Md., were used throughout these studies.

Virus. A Japanese strain (Fu) of CJD virus (27), isolated in mice inoculated with human brain from a patient with an atypical case of CJD, at the second

mouse intracerebral passage level, was used. The inoculum for mice was prepared from mice with advanced clinical CJD. It consisted of the supernatant of a 10% (wt/vol) suspension of brain in phosphate-buffered saline (PBS), pH 7.4, centrifuged at $2,550 \times g$ for 30 min. Thirty microliters (3.1×10^4 mouse 50% lethal doses [LD_{50}]) of this supernatant were inoculated intracerebrally (i.c.) into each weanling mouse.

Removal of infected tissues. Mice were sacrificed at various time intervals as described below. To avoid cross-contamination of tissues, aseptic techniques were employed, and each tissue was processed with a separate set of sterile instruments. Tissues were removed in the following order: urine, blood, brain, spleen, kidney, liver, thymus, and lung. The urine was collected in sterile tubes by pressing the lower abdomen and dialyzed against PBS overnight because of its toxicity. Whole blood was collected by cardiac puncture into heparinized syringes, transferred to a sterile tube, immediately centrifuged at $259 \times g$ for 20 min, and then frozen at -70°C overnight (15). The following day, frozen blood was cut with a sterile blade approximately 0.5 cm beyond each side of the buffy coat. Tissues and body fluids were stored at -70°C until used.

Preparation of spleen cell suspension. Groups of 10 mice were sacrificed at 9 and 18 weeks postinfection. The spleens were removed aseptically, minced finely in ice-cold Eagle minimum essential medium (MEM) containing 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 20% fetal bovine serum (FBS) (20% FBS-MEM), and filtered through several layers of sterile gauze. Erythrocytes were lysed with 0.83% ammonium chloride. The spleen cells were then washed twice and suspended at a concentration of 10^7 cells per ml in 20% FBS-MEM.

Separation of spleen cells. Spleen cells were separated into macrophages and lymphocytes by the differential adherence of cells to plastic. Five milliliters of the

[†] On leave from the Department of Virology, School of Medicine, Kyushu University, Fukuoka, Japan 812 (Present address: Department of Internal Medicine, Saga Medical School, Saga, Japan 840-01).

spleen cell suspension was incubated in a plastic petri dish (Falcon no. 1007; Falcon Plastics, Oxnard, Calif.) at 37°C for 60 min. Plastic-nonadherent cells (lymphocytes) were removed by pipetting. Plastic-adherent cells (macrophages) were collected by a rubber policeman, washed twice, and suspended in 20% FBS-MEM. The purity of the macrophage fraction was examined by the phagocytic activity. Samples (0.5 ml) of a 5% (vol/vol) opsonized sheep erythrocyte suspension were added to the adherent cells in a plastic petri dish and incubated at 37°C for 120 min. They were then rinsed with PBS, and sheep erythrocyte-ingested cells were determined by staining with Giemsa. More than 95% of cells in this fraction were macrophages. A part of the macrophage fraction was cultured for the study of infectivity. They were maintained in plastic petri dishes (Falcon no. 1007) at 37°C in a humidified 5% CO₂ atmosphere, and the medium was changed twice a week. The medium consisted of Dulbecco MEM supplemented with 10% L-cell conditioned medium (19), 10% PBS, and 5% horse serum.

Fractionation of spleen lymphocytes. The plastic non-adherent cells (lymphocytes) were fractionated into T-cell and B-cell fractions by the nylon wool method (16). Briefly, 2×10^8 cells in 2 ml of 20% FBS-MEM were allowed to incubate for 60 min at 37°C in a 20-ml syringe containing 1.5 g of nylon wool. Nylon wool-nonadherent cells were eluted by washing the nylon wool with 60 ml of warm medium; nylon wool-adherent cells were collected by gently squeezing the nylon wool. The efficiency of fractionation was monitored by assaying for T cells by the cytotoxicity test. Briefly, 0.2 ml (2×10^5 cells) of the cell suspension were mixed with 0.2 ml of rabbit anti-mouse thymocyte serum ($\times 256$; Microbiological Associates, Rockville, Md.) and 0.4 ml of guinea pig complement ($\times 5$; Flow Laboratories, Inc., Rockville, Md.) and incubated at 37°C for 60 min. The percentage of T cells in each cell fraction was more than 95% in the T-cell fraction and less than 20% in the B-cell fraction.

Subfractionation of spleen T cells and B cells. Both T-cell and B-cell fractions were further fractionated into 10 subfractions on continuous density gradients of colloidal silica (Percoll; Pharmacia Fine Chemicals, Piscataway, N.J.). A stock solution of Percoll was first made by mixing undiluted Percoll with 0.15 M NaCl at a ratio of 55:25, and 9 ml of this mixture was centrifuged in a fixed-angle rotor at $20,000 \times g$ for 30 min. After centrifugation, 10^8 cells in 1 ml of 0.15 M NaCl was carefully layered on the top of the gradient and centrifuged at $800 \times g$ for 15 min. Ten 1.0-ml fractions were recovered from the top of gradient and analyzed for density profile and cell count.

Lymphocyte culture. The cells from each lymphocyte fraction and subfraction were cultured in microtiter plates (Falcon no. 3040) for the study of DNA synthesis and the proliferative response to lymphocyte mitogens. Cultures were triplicate 0.2-ml samples of the cell suspension containing 2×10^6 cells per ml in 10% FBS-MEM supplemented with 5×10^{-5} M 2-mercaptoethanol. Cells were incubated at 37°C for 3 days in a humidified 5% CO₂ atmosphere and pulsed with 0.4 μ Ci of [³H]thymidine [6-³H]thymidine, 22 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for 24 h before harvesting. For mitogenic stimulation, 20 μ l of concanavalin A (ConA, 20 μ g/ml; Miles Laboratories, Inc., Elkhart, Ind.), or 20 μ l of

lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (20 μ g/ml; Difco Laboratories, Detroit, Mich.) were added to 0.2 ml of the cell suspension. Acid-insoluble materials from cultured cells were collected on glass fiber filters, and isotope incorporation was measured in a liquid scintillation counter.

In vitro infection of normal spleen cells with virus. Virus for these experiments was prepared as follows. Forty milliliters of a 10% (wt/vol) suspension of infected mouse brain was mixed with 20 ml of Freon 113 (dichloro-difluoromethane; Matheson Gas Instruments, Palo Alto, Calif.) and homogenized in a Sorvall Omni-Mixer (Dupont Instruments, Newtown, Conn.) at the top speed (4,000 rpm) for 10 min. This homogenate was subsequently centrifuged at $50,000 \times g$ for 60 min. The aqueous supernatant (3×10^5 mouse LD₅₀ of virus per ml) was used as the source of virus.

The spleen cells were taken from normal BALB/c mice aged 9 weeks and separated into macrophage, T-cell, and B-cell fractions. Part of the T-cell fractions and part of the B-cell fractions were stimulated in vitro with ConA and LPS, respectively. For stimulation of T cells and B cells, 4 μ g of ConA or 40 μ g of LPS was added to each cell suspension (4×10^6 cells per 2 ml) and cultured for periods ranging from 1 to 3 days. Unstimulated and stimulated spleen cells were adjusted to a concentration of 2.5×10^7 cells per 0.5 ml, and equal volumes of Freon-extracted virus solution were mixed with them. They were then incubated at 37°C for 120 min. After incubation, the cells were washed five times with PBS to remove the unadsorbed virus. As a control, a normal spleen lymphocyte suspension and the virus solution were incubated separately at 37°C for 120 min. They were then mixed and, immediately thereafter, washed five times with PBS.

Assay of infectivity of tissues and cells. Individual tissues from CJD virus-infected mice were homogenized in Tenbroeck grinders to a 10% (wt/vol) suspension in PBS and centrifuged in the cold (4°C) at $2,550 \times g$ for 30 min. Thirty microliters of the supernatants was inoculated i.c. into each weanling recipient mouse. The blood and the urine were inoculated i.c. undiluted. To demonstrate viremia, a single dose of 30 μ l of the buffy coat-enriched fraction was inoculated i.c., and 100 μ l of other fractions (serum and erythrocytes) collected at weekly intervals were inoculated intraperitoneally. Once every week for 5 weeks, similarly dialyzed urine, collected on a weekly basis from the same mice, was inoculated i.c. at a dose of 30 μ l.

Each spleen cell fraction taken from CJD virus-infected mice or cells infected in vitro with CJD virus was adjusted at an appropriate cell concentration in PBS as described below. After treatment of three cycles of freezing and thawing, 30 μ l of each cell suspension was inoculated i.c. into each weanling mouse.

After 100 days, mice were examined three times a week for overt signs of the disease, and the examination was continued up to 360 days after inoculation; then asymptomatic survivors were killed for histological study of the CNS. Incubation periods were calculated from the date when unmistakable signs of CJD became apparent. Infectivity was quantitated by the length of mean incubation periods and the incidence of dead mice or by determining the amount of virus in animal tissues or in vitro-infected spleen cells required for 1 mouse LD₅₀.

TABLE 1. Infectivity of tissues from BALB/c mice inoculated i.c. with 3.1×10^4 mouse LD₅₀ of CJD virus^a

| Tissue | Infectivity at week after inoculation of virus: | | | | | | |
|--------|---|-----------|-----------|-----------|-----------|-----------|-----------|
| | 1 | 2 | 3 | 6 | 9 | 14 | 18 |
| Brain | 174 (5/5) | 167 (6/6) | 151 (7/7) | 133 (5/5) | 129 (5/5) | 122 (7/7) | 112 (7/7) |
| Spleen | 167 (6/6) | 133 (6/6) | 129 (6/6) | 126 (6/6) | 126 (6/6) | 136 (6/6) | 151 (6/6) |
| Lung | | | 154 (5/6) | 139 (6/6) | 146 (6/6) | 149 (6/6) | 186 (6/6) |
| Liver | —(0/7) | —(0/7) | —(0/7) | —(0/7) | —(0/7) | —(0/7) | 271 (5/5) |
| Kidney | —(0/7) | —(0/7) | —(0/7) | —(0/7) | 190 (5/7) | —(0/7) | —(0/7) |
| Thymus | | | | | 153 (6/6) | 162 (3/3) | 181 (5/5) |
| Blood | —(0/4) | —(0/4) | —(0/2) | 281 (5/5) | 213 (3/3) | 156 (3/3) | 142 (5/5) |
| Urine | —(0/3) | —(0/4) | —(0/3) | —(0/4) | —(0/6) | —(0/6) | —(0/5) |

^a For the infectivity assays, 0.03 ml of the supernatant of a 10% (wt/vol) homogenate of each tissue was inoculated i.c. into weanling mice; to demonstrate viremia, 0.03 ml was inoculated intraperitoneally into mice; 0.03 ml of urine, previously dialyzed against PBS, was inoculated i.c. The results are expressed as the mean incubation days (—, no infectivity) in the recipient mouse, with the number of dead mice/number of mice examined within parentheses. The standard error was >2.5% of the mean incubation period.

RESULTS

Clinical and histological observation of CJD virus-infected mice. Groups of 7 to 10 mice were killed for the study of infectivity of tissues and histology of the CNS at 1, 2, 3, 6, 9, 14, and 18 weeks after inoculation of virus. One group of 10 mice was used for clinical observation. This group of mice exhibited typical signs of the disease such as ruffled fur, weight loss, arched back, plasticity of tail, and bradykinesia at approximately week 16 postinoculation; the mean incubation period in this group was 112 days, with a duration of clinical disease less than 2 weeks.

The characteristic histopathological changes consisting of status spongiosis, astrocytic proliferation and hypertrophy, and neuronal loss were first noted in the CNS at week 9 postinoculation.

Infectivity of tissues from CJD virus-infected mice. Infectivity was detected in the brain and spleen from week 1 after inoculation of virus (Table 1). Of tissues examined, the highest infectivity was present in the brain or the spleen throughout the infection. However, the mode of replication of virus in the spleen was quite

different from that in the brain. The infectivity of spleen reached its peak before the appearance of clinical disease and decreased remarkably thereafter; meanwhile, the infectivity of brain increased with time and reached its plateau approximately 18 weeks after inoculation, when all surviving mice were moribund. Notably, the highest infectivity was present in the spleen from week 2 through week 9 postinoculation even when the virus was inoculated i.c. Of other tissues examined, moderately high infectivity was found in the lung from week 3 postinfection and in the thymus from week 9 postinfection (Table 1). Low infectivity was detected transiently in the kidney at week 9 and later in the liver at week 18 post-inoculation. Infectivity was present in the circulating blood from the sixth week and persisted throughout the infection. Infectivity was not detected in the urine.

Infectivity of spleen cells from CJD virus-infected mice. Initially, the infectivity of spleen macrophages, T cells, and B cells was studied at the week 18 after inoculation. In this experiment, a dose of 2×10^6 cells was inoculated i.c. into each recipient mouse. Infectivity was found in each of the cell subpopulations (Table 2). How-

TABLE 2. Infectivity of spleen cell subpopulations taken from BALB/c mice with clinical CJD^a

| Cells | Dose (cells/mouse) ^b | No. of mice dead/total | Mean incubation (days) \pm SD |
|---|---------------------------------|------------------------|---------------------------------|
| T cell-enriched fraction | 2×10^6 | 7/7 | 183 \pm 2 |
| B cell-enriched fraction | 2×10^6 | 7/7 | 184 \pm 3 |
| Macrophage-enriched fraction | 2×10^6 | 9/9 | 227 \pm 3 |
| Macrophages cultures in vitro for 4 weeks | 2×10^6 | 7/9 | 276 \pm 3 |
| Macrophages cultures in vitro for 8 weeks | 2×10^6 | 0/9 | |

^a Each spleen cell subpopulation was prepared from mice 18 weeks after an i.c. inoculation of 3.1×10^4 mouse LD₅₀ of virus.

^b Cells were inoculated i.c. after three cycles of freezing and thawing.

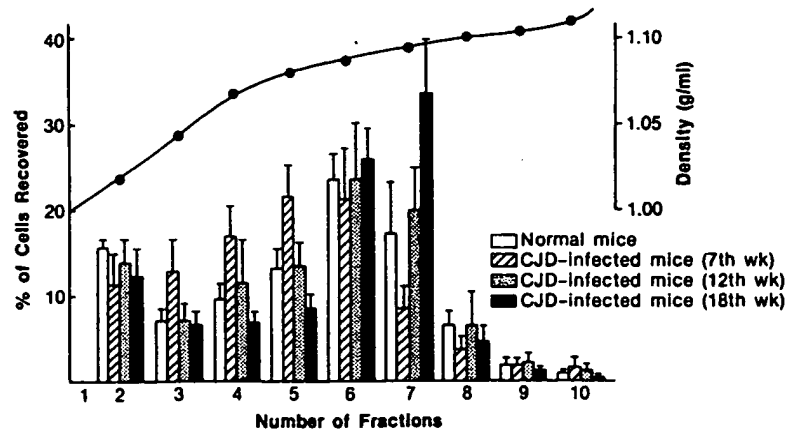


FIG. 1. Density and percentage of spleen cells recovered in each fraction of a continuous density gradient of Percoll. The cells were taken from mice at week 7, 12, and 18 after an i.c. inoculation of 3.1×10^4 mouse LD₅₀ of CJD virus and from normal mice at the same age. Ten 1.0-ml cell fractions were recovered and numbered from the top of gradient. The pattern of distribution of cells throughout the gradient was essentially identical among normal mice with different ages.

ever, the mean incubation period for mice inoculated with the macrophage fraction was delayed more than 40 days from those of mice inoculated with lymphocyte fractions. The highest concentration of virus was present in lymphocyte fractions. The infectivity of macrophages was also studied after 4 and 8 weeks of *in vitro* culture. Infectivity was found in the macrophages after 4 weeks of culture, but the mean incubation period for the recipient mouse was prolonged more than 50 days from that of mice inoculated with the uncultured macrophages (Table 2). Infectivity was not detected in the macrophages after 8 weeks of culture.

Infectivity of spleen lymphocyte subpopulations from CJD virus-infected mice. In an attempt to determine which of the spleen lymphocyte subpopulations were most infectious, spleen lymphocytes obtained from CJD virus-infected mice at week 18 postinoculation were separated into 10 fractions on a continuous density gradient of Percoll. The density profile of Percoll gradient and the percentage of lymphocytes recovered in each 1.0-ml fraction are shown in Fig. 1. Viable cells were not found in the superficial fraction (fraction 1), which consisted of 0.15 M NaCl layered on the top of gradient, whereas the viability of the cells was almost 100% in other fractions. This result showed that dead cells did not enter the gradient of Percoll. The pattern of distribution of cells recovered in each fraction of Percoll gradient in CJD virus-infected mice was almost similar with that of normal mice of the same age (Fig. 1).

The infectivity of cells from each cell fraction could only be tested by the i.c. inoculation of 10^5 cells per mouse because of low recoveries of

cells in fractions 9 and 10 (Fig. 1). The infectivity was found in all fractions except fraction 9 (Table 3). It is evident that lymphocytes from lower-density fractions (fractions 2 to 5; densities, 1.03 to 1.08 g/ml, respectively) were more infectious than those from higher-density fractions (fractions 6 to 10; densities, 1.09 to 1.12 g/ml, respectively). The highest infectivity was found in lymphocytes from fraction 3 (density, 1.05 g/ml) and fraction 4 (density, 1.07 g/ml) as shown by both the shortest mean incubation periods and the highest incidences of dead mice in the recipient mice (Table 3). Morphologically, large lymphocytes or blastoid cells were observed in lower-density fractions, and small lymphocytes were observed in higher-density fractions. This observation was confirmed by the experiment on the lymphocyte thymidine uptake. The level of [³H]thymidine uptake of lymphocytes from fractions 2 to 5 was higher than that of lymphocytes from fractions 6 to 10 (Table 3). Furthermore, the fractionation of *in vitro* mitogen-activated spleen T and B cells from normal mice by Percoll showed that 90% of activated lymphocytes were included in fractions 2 to 5; fractions 3 and 4 contained more than 70% of these activated cells (Fig. 2).

Infectivity of spleen T cells and B cells from CJD virus-infected mice. The infectivity of spleen T cells and B cells from CJD virus-infected mice were further analyzed by the density separation with Percoll. This experiment was performed in mice at week 9 after inoculation when the spleen contained the highest infectivity throughout the infection. In these experiments two serial 1.0-ml fractions were mixed and tested for their infectivity because of very

TABLE 3. Infectivity of spleen lymphocyte subpopulations taken from BALB/c mice with clinical CJD^a

| Lymphocyte fraction | Infectivity ^b | | [³ H]thymidine uptake of cells ^c (cpm) | | |
|---------------------|--------------------------|---------------------------------|---|------------------|--------|
| | No. of mice dead/total | Mean incubation (days) \pm SD | Unstimulated | Stimulated with: | |
| | | | | ConA | LPS |
| Unfractionated | 5/8 | 221 \pm 9 | 706 | 50,072 | 12,584 |
| Fraction 1 | | | | | |
| Fraction 2 | 7/8 | 225 \pm 25 | 640 | 93,716 | 18,763 |
| Fraction 3 | 8/8 | 190 \pm 7 | 2,397 | 46,960 | 11,021 |
| Fraction 4 | 8/8 | 183 \pm 2 | 5,460 | 87,692 | 27,852 |
| Fraction 5 | 6/8 | 221 \pm 5 | 1,108 | 106,126 | 28,142 |
| Fraction 6 | 4/8 | 226 \pm 4 | 507 | 94,425 | 14,512 |
| Fraction 7 | 2/8 | 242 \pm 9 | 135 | 105,430 | 5,942 |
| Fraction 8 | 1/8 | 216 \pm 0 | 104 | 66,740 | 2,608 |
| Fraction 9 | 0/8 | | 470 | 57,988 | 2,101 |
| Fraction 10 | 1/7 | 248 \pm 0 | 199 | 67,456 | 2,134 |

^a Spleen lymphocytes were prepared from mice 18 weeks after an i.c. inoculation of 3.1×10^4 mouse LD₅₀ of virus. Spleen lymphocytes were separated into 10 fractions on a continuous density gradient of Percoll. Fractions were numbered from the top of the gradient.

^b Lymphocytes were inoculated i.c. into the recipient mouse as a concentration of 10^5 cells per mouse after three cycles of freezing and thawing.

^c Lymphocytes were harvested after 3 days of culture. For mitogenic stimulation 0.4 μ g of ConA or 4 μ g of LPS was added to each cell culture (4×10^5 cells per 0.2 ml).

low recoveries of cells in fractions 9 and 10 (Table 4). Infectivity was found in both T-cell and B-cell fractions, but much higher infectivity was found in the B-cell fraction. Evidence of higher infectivity of the B-cell fraction than the T-cell fraction was shown by shorter mean incubation periods, higher incidences of dead mice, and the lesser number of cells required for mouse LD₅₀ in the recipient mice (Table 4). The highest infectivity was found in lower-density B-cell fractions (fractions 2 to 4; densities, 1.03 to 1.07 g/ml, respectively). In the case of T cells, cells from lower-density fractions were also more infectious than those from higher-density fractions.

Concurrently, the mitogenic ability of these infected spleen T cells and B cells was studied (Table 4). The background [³H]thymidine uptake was much higher in cells from the B-cell fraction than those from the T-cell fraction. The highest background [³H]thymidine uptake was found in cells from fractions 3 and 4 of the B-cell population, in which the highest infectivity was also detected. In both cell fractions, cells from lower-density fractions incorporated [³H]thymidine more actively than those from higher-density fractions. The proliferative response of spleen T cells and B cells to each mitogen was well preserved. There was no difference in the level of background [³H]thymidine uptake and proliferative response to mitogens between normal spleen cells and CJD virus-infected ones.

In vitro infection of normal spleen cells with the virus. The susceptibility of spleen cells to CJD virus was also studied by means of in vitro

coincubation of cells with the virus for 120 min at 37°C. In this experiment, unstimulated macrophages, unstimulated T cells and B cells, and in vitro mitogen-activated T cells and B cells were studied for their susceptibility to the virus. Table 5 shows the difference in the quantity of virus present in each cell fraction. Macrophages and unstimulated T cells and B cells required approximately 10^5 cells for one mouse LD₅₀, whereas with 3-day activated T cells and B cells fewer than 10^3 cells were required for one mouse

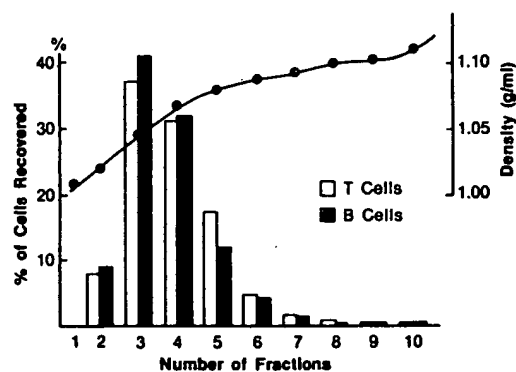


FIG. 2. Density and percentage of mitogen-activated spleen T cells and B cells recovered in each fraction of a continuous density gradient of Percoll. The cells were taken from normal BALB/c mice. For mitogenic stimulation 4 μ g of ConA or 40 μ g of LPS was added to each T-cell or B-cell culture (4×10^6 cells per 2 ml). After 3 days of culture, cells were fractionated into 10 1.0-ml fractions with Percoll gradient. Fractions were numbered from the top of gradient.

TABLE 4. Infectivity and thymidine uptake of spleen T cells and B cells taken from CJD virus-infected BALB/c mice^a

| Spleen lymphocyte subpopulation | Infectivity of cells ^b | | | [³ H]thymidine uptake of cells ^c (cpm) | | |
|---------------------------------|-----------------------------------|-----------------------------|--|---|------------------|--------|
| | No. of mice dead/total | Mean incubation (days) ± SD | No. of cells for mouse LD ₅₀ (log ₁₀) | Unstimulated | Stimulated with: | |
| | | | | | ConA | LPS |
| T cells | | | | | | |
| Unfractionated | 5/6 | 233 ± 17 | 4.0 | 490 | 87,054 | |
| Fractions 1 and 2 | 5/7 | 219 ± 4 | 4.0 | 342 | 54,674 | |
| Fractions 3 and 4 | 7/7 | 227 ± 17 | 4.3 | 785 | 99,333 | |
| Fractions 5 and 6 | 2/6 | 239 ± 5 | 5.0 | 313 | 74,128 | |
| Fractions 7 and 8 | 1/7 | 250 ± 0 | 5.0 | 257 | 59,676 | |
| Fractions 9 and 10 | 0/7 | | | 172 | 29,013 | |
| B cells | | | | | | |
| Unfractionated | 16/18 | 203 ± 15 | 2.6 | 1,091 | | 27,270 |
| Fractions 1 and 2 | 5/7 | 211 ± 15 | 2.6 | 2,181 | | 23,274 |
| Fractions 3 and 4 | 6/7 | 181 ± 10 | 2.6 | 5,473 | | 63,604 |
| Fractions 5 and 6 | 6/7 | 212 ± 17 | 3.5 | 1,944 | | 44,878 |
| Fractions 7 and 8 | 3/6 | 224 ± 2 | 5.0 | 350 | | 16,787 |
| Fractions 9 and 10 | 4/6 | 233 ± 13 | 4.5 | 362 | | 8,568 |

^a Spleen T and B cells were prepared from mice 9 weeks after an i.c. injection of 3.1×10^4 mouse LD₅₀ of CJD virus. They were fractionated into 10 fractions on a continuous density gradient of Percoll. Fractions were numbered from the top of gradient.

^b Mice were inoculated i.c. with 3.3×10^5 cells per mouse.

^c For mitogenic stimulation, 0.4 µg of ConA or 4.0 µg of LPS was added to each cell culture (4×10^5 cells per 0.2 ml).

LD₅₀. No virus was demonstrated in the cells used as controls.

DISCUSSION

The present studies demonstrate the mode of replication and temporal distribution of virus in CJD in mice. Replication of a Japanese strain (Fu) of CJD virus was indistinguishable from that of scrapie virus; in scrapie in mice the virus is present in the brain, spleen, thymus, and lung at high titers and in the kidney and liver at low titers (6, 22). The present studies also demonstrated the persistence of viremia from the early stage of infection in CJD. This result confirmed the earlier observation of Manuelidis et al. (20) in CJD in guinea pigs. The presence of infectivity in the peripheral blood was also reported by our group (12) and others (3) in scrapie and by Tateishi et al. (27) in CJD. In contrast, several investigators have reported their failure to detect infectivity in the blood when whole blood (24) or serum (6) were tested for infectivity. The possibility of contamination of infectious tissues into blood can be ruled out in the present studies because the blood was taken by direct cardiac puncture. The successful demonstration of viremia in the present studies was obtained by serial i.c. inoculation of buffy coat, suggesting that in the peripheral blood maximum titer of virus exists in leukocytes (buffy coat), rather than in the serum or erythrocytes. The persistence of viremia from the early stage of infection in the

present studies would also indicate that the blood is the most likely vehicle for the secondary dissemination of virus from the initial replication sites in CJD.

The highest infectivity from the weeks 2 through 9 post-inoculation was observed in the spleen even when the virus was inoculated i.c. A number of findings can be construed as supporting this result. In scrapie in mice, in addition to the findings of earliest rise in titer in the spleen irrespective of route of infection (6, 22), there is an increased incubation period in splenectomized or spleenless mutant mice by the intraperitoneal route of inoculation (7, 8), suggesting that there is an initial replicative phase of the infection in the spleen. The question therefore arises as to which spleen cell subpopulations are involved in the initial extraneural replicative phase of the infection. The present studies on the infectivity of spleen cells at the early (week 9) and late (week 18) stages of the infection demonstrated that lower-density lymphocytes contained a high concentration of virus. Further, spleen lymphocytes from lower-density fractions of the Percoll gradient were highly enriched in blastoid cells synthesizing DNA actively. Accordingly, it is assumed that developing lymphocytes, i.e., the blastoid cells, play an important role as the initial replication site of virus in the pathogenesis of spongiform encephalopathies. Experiments of in vitro infection of spleen cells with CJD virus supported this view.

TABLE 5. Infectivity of spleen cell subpopulations infected in vitro with CJD virus^a

| Spleen lymphocyte subpopulations | No. of mice dead/total ^b | Mean incubation (days) \pm SD | Amount of cells for mouse LD ₅₀ ^c |
|----------------------------------|-------------------------------------|---------------------------------|---|
| Unstimulated cells | | | |
| Macrophages | 1/4 | 238 \pm 0 | >5.0 |
| Unfractionated lymphocytes | 5/6 | 210 \pm 14 | 4.5 |
| T cells | 6/7 | 207 \pm 17 | 4.4 |
| B cells | 1/7 | 204 \pm 0 | >5.0 |
| 1-day stimulated cells | | | |
| T cells | 5/6 | 195 \pm 3 | 3.9 |
| B cells | 7/7 | 180 \pm 2 | 4.0 |
| 2-day stimulated cells | | | |
| T cells | 7/7 | 168 \pm 2 | 3.6 |
| B cells | 7/7 | 171 \pm 5 | 3.5 |
| 3-day stimulated cells | | | |
| T cells | 7/7 | 170 \pm 7 | 2.7 |
| B cells | 7/7 | 165 \pm 7 | 2.5 |

^a The cells were taken from normal BALB/c mice. For stimulation of T cells and B cells, 4 μ g of ConA and 40 μ g of LPS, respectively, were added to each cell culture (4×10^6 cells per 2 ml). Both unstimulated and stimulated cells (2.5×10^7 cells per 0.5 ml) were coincubated with CJD virus (1.5×10^5 mouse LD₅₀ per 0.5 ml) at 37°C for 120 min. The cells were then washed five times with PBS, adjusted to concentrations of 10^3 to 10^6 cells per 3 ml, and treated with three cycles of freezing and thawing.

^b The incidence of dead mice and mean incubation period in the recipient mouse inoculated intracerebrally with 10^5 cells per mouse.

^c The amount of cells needed for one mouse LD₅₀ (log titer). The cells were inoculated i.c. at concentrations of 10^5 cells per mouse.

The activation of spleen lymphocytes with mitogens enhanced the susceptibility of cells to the virus. Lavelle et al. (18) reported that in scrapie virus-infected mice the highest infectivity was detected in large spleen cells from lower-density fractions of discontinuous albumin density gradients, and the separation of macrophage-enriched fraction did not result in significant enrichment of scrapie virus-infected cells. Moreover, the susceptibility of mice to scrapie was reduced after steroid administration (23), whereas mitogenic stimulation of the host enhanced susceptibility to the disease (4). The mechanisms of these phenomena are not understood, but possible explanations are that these immunological treatments eliminated or increased activated lymphocytes providing the initial replication site for virus. This view is not surprising since it is well known that activated lymphocytes have the capacity to support replication of diverse types of conventional viruses (5, 17, 25, 29).

As for the difference in the infectivity between T cells and B cells in the present studies, one possible explanation for this difference could be that genetically there is a higher susceptibility of B cells to CJD virus. However, the experiments of in vitro infection of spleen cells with the virus showed that there was no difference in susceptibility between T cells and B cells. The most probable explanation for this difference is that

lower-density B cell fractions were highly enriched in blastoid cells having the capacity to support virus replication.

There exists a possibility that the contamination of free virus or dead cells introduced artifacts in the infectivity titer of each cell fraction; however, the densities associated with the highest infectivity, i.e., 1.05 to 1.07 g/ml, were absolutely different for the findings of buoyant density studies of cell-free scrapie virus showing peak infectivity at densities of 1.14 to 1.30 g/ml (2, 26). Concerning the possible contamination of dead cells into each cell fraction, we demonstrated that dead cells did not enter the gradient of Percoll. Gmelig-Meyling and Waldman (14) also reported no entrance of dead cells into Percoll gradients. Therefore, we assume that the difference in the titer of infectivity resulted from the difference in specific infectivity of each cell fraction.

Recently, Garfin et al. (10, 11) reported that the proliferative response of spleen lymphocytes to LPS was significantly suppressed in scrapie virus-infected BALB/c and C3H/HeJ mice. Their findings suggest that this unconventional virus has a capacity to exert a biological influence on host cells. To test this hypothesis, the responsiveness of CJD virus-infected spleen lymphocytes to mitogens was analyzed in detail. Spleen lymphocytes were fractionated into T cells and B cells and thereafter further fraction-

ated into five fractions by continuous density gradients of Percoll. The results of these experiments showed that CJD virus did not exert any biological influence on the ability of T cells and B cells to respond to mitogens. Our previous experiments of coinfection of normal spleen cells with CJD virus in the presence of ConA or LPS for periods ranging from 2 to 5 days also showed that the virus exerted no influence on the mitogen-driven lymphocyte proliferative response.

The strain of CJD virus used in the present studies is different from other strains in that this strain was associated with considerable status spongiosis of the white matter of the patient's brain as well as the brains of experimentally infected animals, and it could be transmitted into small rodents (27, 28). However, fundamental physical and chemical properties and biological behaviors are essentially the same with other strains of CJD virus and scrapie virus (27, 28). Therefore, the general phenomenon in the pathogenesis of CJD in mice may be identical to that in primates. Accordingly, of particular significance to our understanding of CJD infection in humans is the finding of the wide dissemination and persistence of high concentrations of virus in extraneural tissues, including blood, long before the onset of clinical disease.

LITERATURE CITED

1. Asher, D. M., C. J. Gibbs, Jr., and D. C. Gajdusek. 1976. Pathogenesis of subacute spongiform encephalopathies. *Ann. Clin. Lab. Sci.* 6:84-103.
2. Brown, P., E. M. Green, and D. C. Gajdusek. 1978. Effect of different gradient solution on the buoyant density of scrapie infectivity. *Proc. Soc. Exp. Biol. Med.* 158:513-516.
3. Clarke, M. C., and D. A. Haig. 1967. Presence of the transmissible agent of scrapie in the serum of affected mice and rats. *Vet. Rec.* 80:504.
4. Dickinson, A. G., H. Fraser, I. McConnele, and G. W. Outram. 1978. Mitogenic stimulation of the host enhances susceptibility to scrapie. *Nature (London)* 272:54-55.
5. Edelman, R., and E. E. Wheelock. 1966. Vestibular stomatitis virus replication in human leukocyte cultures: enhancement by phytohemagglutinin. *Science* 154:1053-1055.
6. Eklund, C. M., R. C. Kennedy, and W. J. Hadlow. 1967. Pathogenesis of scrapie virus infection in the mouse. *J. Infect. Dis.* 117:15-22.
7. Fraser, H., and A. G. Dickinson. 1970. Pathogenesis of scrapie in the mouse: the role of spleen. *Nature (London)* 226:462-463.
8. Fraser, H., and A. G. Dickinson. 1978. Studies of the lymphoreticular system in the pathogenesis of scrapie: the role of spleen and thymus. *J. Comp. Pathol.* 88:563-573.
9. Gajdusek, D. C. 1977. Unconventional viruses and the origin and disappearance of kuru. *Science* 197:943-960.
10. Garfin, D. E., D. P. Stites, J. D. Perlman, S. P. Cochran, and S. B. Prusiner. 1978. Mitogenic stimulation of splenocytes from mice infected with scrapie agent. *J. Infect. Dis.* 138:396-400.
11. Garfin, D. E., D. P. Stites, L. A. Zitnik, and S. B. Prusiner. 1978. Suppression of polyclonal B cell activation in scrapie-infected C3H/HeJ mice. *J. Immunol.* 120:1986-1990.
12. Gibbs, C. J., Jr., and D. C. Gajdusek. 1978. Subacute spongiform virus encephalopathies: transmissible virus dementia. *Aging* 7:559-575.
13. Gibbs, C. J., Jr., D. C. Gajdusek, and C. L. Masters. 1978. Considerations of transmissible subacute and chronic infections, with a summary of the clinical and virological characteristics of kuru, Creutzfeldt-Jakob disease and scrapie, p. 115-130. *In* K. Nandy (ed.), *Senile dementia: a biomedical approach*. Elsevier/North-Holland Biomedical Press, New York.
14. Gmellig-Meyling, F., and T. A. Waldman. 1980. Separation of human blood monocytes and lymphocytes on a continuous Percoll gradient. *J. Immunol. Methods* 33:1-9.
15. Horstmann, D. M. 1952. Poliomyelitis virus in blood of orally infected monkeys and chimpanzees. *Proc. Soc. Exp. Biol. Med.* 79:417-419.
16. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645-649.
17. Kleiman, L. F., S. Kibrick, F. Ennis, and P. Polgar. 1972. Herpes simplex virus replication in human lymphocyte cultures stimulated with phytohemagglutinin and anti-lymphocyte globulin. *Proc. Soc. Exp. Biol. Med.* 141:1095-1099.
18. Lavelle, G. C., L. Sturman, and W. J. Hadlow. 1972. Isolation from mouse spleen of cell populations with high specific infectivity of scrapie virus. *Infect. Immun.* 5:319-323.
19. Manuel, V., and V. Defendi. 1971. Regulation of DNA synthesis in mouse macrophages. I. Sources, action and purification of the macrophage growing factor (MGF). *Exp. Cell Res.* 65:33-42.
20. Manuelidis, E. E., E. J. Gorgacz, and L. Manuelidis. 1978. Viremia in experimental Creutzfeldt-Jakob disease. *Science* 200:1069-1071.
21. Marsh, R. F., J. M. Miller, and R. P. Hanson. 1973. Transmissible mink encephalopathy: studies on the peripheral lymphocyte. *Infect. Immun.* 7:352-355.
22. Outram, G. W. 1976. The pathogenesis of scrapie in mice, p. 325-357. *In* R. H. Kimberlin (ed.), *Slow virus diseases of animals and man*. North-Holland Publishing Co., Amsterdam.
23. Outram, G. W., A. G. Dickinson, and H. Fraser. 1974. Reduced susceptibility to scrapie in mice after steroid administration. *Nature (London)* 249:855-856.
24. Parrison, I. H., and G. C. Milson. 1962. Distribution of the scrapie agent in the tissues of experimental inoculated goat. *J. Comp. Pathol.* 72:233-244.
25. Paul, P. S., W. L. Mengeling, and T. T. Brown, Jr. 1979. Replication of porcine parvovirus in peripheral blood lymphocytes, and peritoneal macrophages. *Infect. Immun.* 25:1003-1007.
26. Siakotos, A. N., D. C. Gajdusek, C. J. Gibbs, Jr., R. D. Traub, and C. Bucana. 1976. Partial purification of the scrapie agent from mouse brain by pressure disruption and zonal centrifugation in sucrose-sodium chloride gradients. *Virology* 70:230-237.
27. Tateishi, J., M. Ohta, M. Koga, Y. Sato, and Y. Kuroiwa. 1980. Transmission of chronic spongiform encephalopathy with kuru plaques and leukomalacia to small rodents. *Ann. Neurol.* 5:581-584.
28. Tateishi, J., Y. Sato, M. Koga, H. Doi, and M. Ohta. 1980. Experimental transmission of human subacute spongiform encephalopathy to small rodents. I. Clinical and histological observations. *Acta Neuropathol.* 51:127-134.
29. Willems, F. T. C., J. L. Melnick, and W. E. Rawls. 1969. Replication of poliovirus in phytohemagglutinin-stimulated human lymphocytes. *J. Virol.* 3:451-457.

treatment did not affect the development of the embryos or their hatching from eggs of normal hens. In addition, the acetone alone did not affect the poor hatchability of the eggs under study (Table 2). The acetone solutions were aseptically placed near the yolk with a Hamilton syringe and the eggs sealed with collodion or Duco cement. All eggs were placed in the same incubator and the results are shown in Table 2. Injection of vitamin D₃ itself markedly improved the hatchability of the eggs. Although injection of either 25-OH-D₃ or 1,25-(OH)₂D₃ improved hatchability of the embryos, at the doses used, hatchability did not return to normal. Nevertheless there is no doubt that injection of all the vitamin D compounds markedly improved embryo development and hatching. These results demonstrate the importance of vitamin D and its metabolites in chick embryonic life and development. They also strongly suggest that 1,25-(OH)₂D₃ is not transferred in adequate amounts to the yolk from the maternal circulation.

Exactly why vitamin D deficiency causes a failure in mandible development is unknown, but improper calcium transport or defective collagen synthesis may be involved. In addition, chick embryos from eggs produced by hens maintained on 1,25-(OH)₂D₃ may provide an important experimental approach to some of the functions of vitamin D. It is also likely that hens maintained on 1,25-(OH)₂D₃ may be used to provide vitamin D deficient embryonic tissue for tissue culture experiments. Certainly these experiments demonstrate that 1,25-(OH)₂D₃ cannot satisfy all of the functions of vitamin D in the laying hen.

M. L. SUNDE, C. M. TURK

Department of Poultry Science,
College of Agricultural and Life
Sciences, University of
Wisconsin-Madison, Madison 53706

H. F. DeLUCA

Department of Biochemistry, College
of Agricultural and Life Sciences,
University of Wisconsin-Madison

References and Notes

1. H. F. DeLuca and H. K. Schnoes, *Annu. Rev. Biochem.* 45, 631 (1976); E. Kodicek, *Lancet* 1974, 326 (1974); A. W. Norman and H. L. Henry, *Rev. Prog. Horm. Res.* 30, 431 (1974).
2. P. C. Miller and M. L. Sunde, *Poultry Sci.* 54, 1422 (1975).
3. The 25-OH-D₃ and 1,25-(OH)₂D₃ were provided, respectively, by F. Frank of Upjohn and M. Usibakov of Hoffmann-La Roche.
4. Crystalline vitamin D₃ was purchased from Phillips Duphar.
5. G. Jones and H. F. DeLuca, *J. Lipid Res.* 16, 440 (1975).
6. M. F. Holick and H. F. DeLuca, *Adv. Steroid Biochem. Pharmacol.* 4, 111 (1974).
7. This work was supported by NIH program-project grant AM-14881, Hatch Act project No. 302, and the Harry Steenbock Research Fund.

3 December 1977; revised 6 March 1978

SCIENCE, VOL. 200, 2 JUNE 1978

Viremia in Experimental Creutzfeldt-Jakob Disease

Abstract. Inoculation of the buffy coat of blood from guinea pigs infected with Creutzfeldt-Jakob disease resulted in passage of this disease to recipient animals. These findings suggest that the hematogenous route may be implicated in the human infection and that the disease may possibly be transmitted by blood transfusions.

The occurrence of viremia in encephalopathies caused by conventional viruses is a well-recognized event, and its importance in the pathogenesis of these diseases has been emphasized (1). However, in spongiform virus encephalopathies, which include scrapie of the sheep, transmissible mink encephalopathy, and kuru and Creutzfeldt-Jakob disease of man (2), no viremia has been reported in the diseases affecting humans (3). Similarly, no stages of viremia have been detected in large-scale timed experiments dealing with the pathogenesis of scrapie. Thus, in experiments on the distribution of the scrapie virus in tissues and body fluids during the course of the infection in goats (4, 5) and in mice (6), no virus was found at any time in the circulating blood. However, in a few isolated instances and contrary to these pathogenesis studies, it has been claimed that blood or serum did contain the agent. Thus, serums and blood from mice taken up to 18 hours after inoculation (7), serum of a ram with natural scrapie (8), and serums of mice and rats (9) in the terminal stages of scrapie have been reported to be infective. In one study, the presence of scrapie agent in the blood in a small percentage of mice was thought most likely to result from tissue contamination (10).

We have transmitted Creutzfeldt-Jakob disease to guinea pigs and serially propagated it (11, 12). Using the guinea pig model, we undertook a series of

timed experiments in order to study the pathogenesis of experimental Creutzfeldt-Jakob disease by virological and light and electron microscopic techniques. Our findings indicate the presence of viremia in this disease.

We inoculated 140 approximately 3-month-old guinea pigs (Hartley strain) with 0.1 ml intracerebrally of a 10⁻² suspension of brain in normal saline from two guinea pigs that developed Creutzfeldt-Jakob disease during the fifth serial passage. Starting with the 1st week and at weekly intervals, up to the 28th week after inoculation, two inoculated guinea pigs (donors) were anesthetized with ether and the thorax was opened sterily; then 8 ml of blood was removed, with a heparinized syringe, from the heart of each animal. After the blood was removed, the animals were killed and, in addition to the blood, various tissues including the central nervous system (CNS) were removed for virological and microscopic examinations. In our attempt to demonstrate the presence of viremia, we used the technique described by Horstmann (13). The blood removed from each animal was placed in a sterile polyallomer tube within a larger sterile polypropylene tube, centrifuged at 1000 rev/min for 15 to 20 minutes and frozen at -90°C overnight. The following day, each frozen tube was cut with a sterile blade approximately 0.5 cm beyond each side of the buffy coat. The section of the blood containing the entire



Fig. 1. Thalamus from animal inoculated with blood drawn at week 25. Several disintegrating neurons are seen (arrows) in a field showing spongiform changes of the surrounding neuropil. Hematoxylin-eosin (x 640).

buffy coat and 0.5 ml each of plasma and red blood cells were placed in a sterile glass tube and thawed. The buffy coat was used for inoculation undiluted. Three normal guinea pigs (recipients) were inoculated with the blood of each donor animal every week; each guinea pig received 0.1 ml intracerebrally, subcutaneously, intramuscularly, and intraperitoneally.

Virus was present in the circulating blood drawn from donor guinea pigs at the 1st, 2nd, 3rd, 12th, 13th, 15th, 20th, 24th, 25th and 26th weeks after inoculation (Table 1). The brains of recipients inoculated with these bloods showed microscopic evidence of a spongiform virus encephalopathy, which is characteristic for experimental Creutzfeldt-Jakob disease. The fate of many recipient animals is still pending; thus it cannot yet be decided conclusively

whether there is an eclipse of the virus from the blood of the donor guinea pigs—for example, between the 2nd and 12th weeks. By the 28th week remaining donor animals showed clinically and microscopically advanced stages of experimental Creutzfeldt-Jakob disease.

In contrast to signs in the donor animals that were inoculated with infected brain, the clinical signs of the disease in the recipient guinea pigs inoculated with the buffy coat were very subtle, consisting of ruffled fur, sluggish or uncoordinated movements of the extremities and head, lack of interest in food, and generalized weakness. Ten of the positive recipient animals showed these clinical signs (14), and, with the exception of the CNS lesions, no pathological findings were present in the visceral organs. The long incubation periods in

these animals suggest that the titers of virus in the blood were low. In the remaining four positive recipient animals, none of the above clinical signs could be detected; within a day or two these animals became prostrated and moribund without any detectable prodromal signs (15).

The recipient animals were defined as positive by the presence of a spongiform encephalopathy. The most conspicuous and prominent change was a widespread destruction of the nerve cells, which was present predominantly in the cerebral cortex, and to a lesser degree in the basal nuclei, thalamus, and hypothalamus. In some cortical regions only a few well-preserved neurons were present, many nerve cells had disappeared, and in others the only structure still remaining was the swollen, pale, lytic nucleus. Status spongiosus was seen in the neuropil (Fig. 1), and to a lesser degree vacuoles were found in the cytoplasm of the nerve cells in the cortex and in the subcortical gray structures. A moderate increase of astrocytes and mild increase of microglia cells were present along with the neuronal destruction. No inflammatory perivascular or diffuse infiltrates of any type were encountered. Control animals inoculated similarly with blood of normal healthy guinea pigs have not shown any spongiform virus encephalopathy.

The successful demonstration of viremia in our pathogenesis experiments is attributed to the technique used. In studying the temporal distribution of the virus in tissues and body fluids on animals inoculated with scrapie, no virus was found in the circulating blood, when whole blood (4), blood clot, or serum (5, 6) were studied for infectivity. On the basis of the results of Eklund *et al.* (6) which showed that scrapie replicates in "lymphocytic tissues" (spleen, lymph nodes, thymus) after subcutaneous inoculation of mice, we postulated that maximal infectivity should reside in the buffy coat (white blood cells) rather than with the serum or with red blood cells. Eklund *et al.* showed high virus titers in the lymphocytic tissues by the 8th week and before the virus was detected in the brain by the 16th week after subcutaneous inoculation (6).

In experimental Creutzfeldt-Jakob disease, the demonstration of viremia indicates that this agent may be disseminated by the hematogenous route. Thus, this route appears to be important for the pathogenesis of subacute spongiform virus encephalopathies as it is in encephalitis caused by conventional viruses. Our studies do not exclude the possi-

Table 1. Preliminary data on viremia experiments in Creutzfeldt-Jakob disease.

| Blood taken after infection | Guinea pigs inoculated with blood | Subsequent condition | Spongiform encephalopathy |
|-----------------------------|-----------------------------------|----------------------|---------------------------|
| 1st week | No. 1 | Day 443, killed | + |
| | No. 2 | Pending | |
| | No. 3 | Pending | |
| 2nd week | No. 1 | Day 429, killed | + |
| | No. 2 | Day 488, found dead | |
| | No. 3 | Pending | |
| 3rd week | No. 1 | Day 2, found dead | + |
| | No. 2 | Day 2, found dead | |
| | No. 3 | Day 308, killed | |
| 4th to 11th weeks | Three animals each week | All alive (pending) | |
| 12th week | No. 1 | Day 438, found dead* | + |
| | No. 2 | Day 504, killed | |
| | No. 3 | Pending | |
| 13th week | No. 1 | Day 379, killed | + |
| | No. 2 | Pending | |
| | No. 3 | Pending | |
| 14th week | Three animals each week | All alive (pending) | |
| 15th week | No. 1 | Day 52, found dead* | + |
| | No. 2 | Day 138, found dead* | |
| | No. 3 | Day 463, killed | |
| 16th to 19th weeks | Three animals each week | All alive (pending) | |
| 20th week | No. 1 | Day 396, killed | + |
| | No. 2 | Day 447, killed | |
| | No. 3 | Pending | |
| 21st to 23rd weeks | Three animals each week | All alive (pending) | |
| 24th week | No. 1 | Day 1, found dead | + |
| | No. 2 | Day 307, killed | |
| | No. 3 | Pending | |
| 25th week | No. 1 | Day 147, killed | + |
| | No. 2 | Day 184, killed | |
| | No. 3 | Day 343, killed | |
| 26th week | No. 1 | Day 210, killed | + |
| | No. 2 | Pending | |
| | No. 3 | Pending | |
| 27th and 28th weeks | Three animals each week | All alive (pending) | |

* Autolysis.

bility of a pluripotential spread of the virus in subacute spongiform virus encephalopathies. However, the available results in scrapie—namely, spread of the infection after intracerebral inoculation to visceral tissues (4, 5) and the reverse, spread of the infection from the periphery to the brain (5, 6)—can most easily be explained by viremia rather than by propagation of the virus along neural pathways, either centrifugally and centripetally from and to the brain. The demonstration of viremia in experimental Creutzfeldt-Jakob disease may also have implications for the disease afflicting man.

Although it is not known how the infection spreads in human spongiform virus encephalopathies, the virus of Creutzfeldt-Jakob disease has been found in the liver, kidney, lung, lymph nodes, and cerebral spinal fluid, and the virus of kuru has been found in lymph nodes, kidney, and spleen of humans (16). It is conceivable that the hematogenous spread of the infection is also implicated in man. The presence of virus in the blood in experimental Creutzfeldt-Jakob disease suggests that this may be true and that there may well be a danger of transmitting this disease via blood transfusions from humans harboring the agent during the incubation period, when the clinical disease is not readily apparent. Gajdusek mentioned that two humans harboring Creutzfeldt-Jakob disease were professional blood donors until shortly before the onset of their symptoms (3).

ELIAS E. MANUELIDIS
EDWARD J. GORGACZ,
LAURA MANUELIDIS

Departments of Pathology
and Neurology, Yale University
School of Medicine,
New Haven, Connecticut 06510

References and Notes

1. R. T. Johnson and C. A. Mims, *N. Engl. J. Med.* 278, 23 and 84 (1968).
2. C. J. Gibbs, Jr., and D. C. Gajdusek, *J. Clin. Pathol.* 25 (Suppl. 6), 84 (1972).
3. D. C. Gajdusek, *Science* 191, 943 (1977).
4. I. H. Pattison and G. C. Milson, *J. Comp. Pathol.* 72, 233 (1962).
5. W. J. Hadlow, C. M. Eklund, R. C. Kennedy, T. A. Jackson, H. W. Whidford, C. C. Boyle, *J. Infect. Dis.* 129, 559 (1974).
6. C. M. Eklund, R. C. Kennedy, W. J. Hadlow, *Ibid.* 127, 15 (1967).
7. E. J. Field, E. A. Caspary, G. Joyce, *Vet. Rec.* 83, 109 (1968).
8. C. J. Gibbs, Jr., D. C. Gajdusek, J. A. Morris, in *Slow, Latent and Temperate Virus Infections*, D. C. Gajdusek, C. J. Gibbs, Jr., M. Alpers, Eds., National Institute of Neurological Diseases and Blindness, Monograph No. 2 (Department of Health, Education and Welfare, Washington, D.C., 1965), p. 195.
9. M. C. Clark and D. A. Haig, *Vet. Rec.* 80, 504 (1967).
10. A. G. Dickinson, V. M. H. Meikle, H. Fraser, *J. Comp. Pathol.* 79, 15 (1969).
11. E. E. Manuelidis, *Science* 190, 571 (1975).
12. J. Kim, J. N. Angelo, L. Manuelidis,

Proc. Natl. Acad. Sci. U.S.A. 73, 223 (1976).
M. Horstmann, *Proc. Soc. Exp. Biol. Med.* 417 (1972).
The animals with clinical signs were: No. 1 of the 1st week group, No. 1 of the 2nd week group, No. 3 of the 3rd week group, No. 2 of the 12th week group, No. 3 of the 15th week group, Nos. 1 and 2 of the 20th week group, No. 2 of the 24th week group, and Nos. 2 and 3 of the 25th week group (Table I).
15. Autopsies of these guinea pigs revealed: hemopericardium (No. 2 of the 2nd week group).

stomach dilation and distention of stomach (two of the 1st week group), liver abscesses (No. 1 of the 1st week group), and polycystic kidneys (No. 1 of the 25th week group).
16. D. M. Asher, C. J. Gibbs, Jr., D. C. Gajdusek, *Ann. Clin. Lab. Sci.* 6, 84 (1976).
17. The technical assistance of P. Johnson, M. Von Ehr, and E. Mufly is gratefully acknowledged. This work was supported by grant NS-12674 from the National Institutes of Health.

30 August 1977; revised 20 December 1977

Search Image for Leaf Shape in a Butterfly

Abstract. The butterfly *Battus philenor* forms search images for leaf shape when searching for its two larval host plants in southeast Texas. This behavior increases the rate of discovery of host plants and permits females to track changes in relative host plant suitability for larval growth. Apostatic selection resulting from search image formation is a likely explanation for divergence in leaf shape by the two host plants.

The significance of a plant's leaf shape has been attributed to abiotic environmental factors (1). In contrast, Gilbert (2) noted that the species in some tropical plant families that support populations of coevolved herbivorous insects differ greatly in leaf shape. He suggested that apostatic selection (3, 4) exerted by those herbivores may have produced the observed leaf shape diversity. Central to Gilbert's argument is the assumption that searching insects are able to discriminate leaf shapes and that individuals searching for one leaf type are less likely to respond to another. One mechanism that can lead to such differential response is search image formation. I report here that (i) ovipositing females of the pipevine swallowtail butterfly, *Battus philenor*, search selectively for either broad- or narrow-leaved larval host plants; (ii) females can switch preference from one leaf shape to another on the basis of experience and hence form true search images; (iii) a search image for one leaf shape results in host plants with that leaf shape being discovered in greater proportion than their abundance in the habitat; and (iv) butterflies with strong search images discover larval food plants at higher rates than butterflies with weak search images. In addition, I suggest that *B. philenor* is the primary selective agent responsible for divergence in leaf shape by its two larval host plants in southeast Texas.

I conducted this study between 22 March and 22 May 1977 in the open longleaf pine uplands of the Big Thicket region of southeast Texas. Adults of *B. philenor* are common in the pine upland habitat at that time of year; females can be found searching among the herbaceous vegetation for the two larval food plants *Aristolochia reticulata* and *A. ser-*

pentaria (Aristolochiaceae) (5). The two host plants, perennial herbs reaching a maximum height of 40 to 50 cm, are closely related within the genus *Aristolochia* (6), yet differ in leaf shape. The more common species *A. reticulata* has the broad, ovate leaves characteristic of the genus (6), whereas all *A. serpentaria* plants in areas of sympatry with *A. reticulata* have long, narrow, parallel-sided leaves resembling grass blades.

Ovipositing *B. philenor* can be followed easily in the field, permitting observation of host plant search behavior under natural conditions (5). My preliminary observations suggested that females use leaf shape as a visual cue for locating host plants. As a female flies slowly above the herbaceous vegetation, she periodically approaches and lands on a plant and "tastes" it, presumably with tarsal chemoreceptors similar to those present in other insects (7). If the plant is not an *Aristolochia*, she immediately resumes search flight. If the plant is an *Aristolochia*, she either lays a small cluster of eggs or resumes search flight without ovipositing. Since the two *Aristolochia* species constitute less than 5 percent of the plants that females approach and "taste," it seems unlikely that the butterflies recognize a plant as an *Aristolochia* by employing long-distance olfactory cues such as are used by some other insects (8).

To test the hypothesis that leaf shape is an important cue used in initiating approach to a plant, I observed ovipositing females in an approximately 80-acre (32 ha) area of open longleaf pine upland in the Kirby State Forest, 15 miles (24 km) north of Kountze in Hardin County, Texas. All herbaceous plants and shrubs growing in the area were classified as having either long, narrow leaves or

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.